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(54) Title: PROCESS FOR INDUCING IMMUNOLOGICAL TOLERANCE FOR XENOGENEIC TRANSPLANTS

(57) Abstract: The present invention relates to methods of inducing immunological tolerance in an animal receiving xenogeneic cells and tissues, especially by way of transplant, said methods comprising myeloablative or non-myeloablative conditioning regimens and administration to the recipient of a therapeutically effective amount of a mixture of transforming growth factor-β (TGF-β) antagonists and tumor necrosis factor-α (TNF-α) antagonists. The methods also include the use of anti-T cell antibodies as well as co-stimulatory blockade molecules such as anti-CD40 Ligand antibody and CTLA4lg.

PROCESS FOR INDUCING IMMUNOLOGICAL TOLERANCE FOR XENOGENEIC TRANSPLANTS

This application claims the priority of U.S. Provisional Application 60/160,971, filed 22 October 1999, the disclosure of which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods of enhancing hematopoiesis of donor progenitor cells following xenogeneic transplant, especially into a primate, such as a human.

BACKGROUND OF THE INVENTION

Organ procurement currently poses one of the major problems in solid organ transplantation, as the number of patients requiring transplants far exceeds the number of organs available. A path for eliminating the shortage of donor organs for allotransplantation is to develop the technologies required to transplant non-human organs into humans, i.e., xenotransplantation. The development of clinical xenotransplantation will also allow for the transplantation of non-human cells and tissues.

The species considered to be a primary potential source of such xenogeneic organs is the pig. In particular, a certain strain of the domesticated pig, denoted miniature swine, is considered ideal partly because of its similar size to humans. Furthermore, the proposed use

of pigs as organ donors in xenotransplantation would obviate problems associated with the consideration of non-human primates as donors. For example, xenografts (generally, the transplanting of organs, tissues or cells between animals of different species) from non-human primates to humans present considerable risk of transmission of pathogens and consequent development of emerging infections. Several pathogens that cause disease are known to infect both humans and non-human primates, such as the transmission of HIV from the chimpanzee to humans. Furthermore, chimpanzees and orangutans, the closest non-human primates phylogenetically, are endangered species and far too rare to be considered for organ transplantation purposes. Alternatively, baboons are too small to be an appropriate donor for most organ transplants. Even the largest baboons weigh less than 40 kg. In addition, the gestation times and productivity of primates would not allow a commercially significant generation of source animals.

The physiology of many organ systems of pigs has been shown to be highly similar to the human counterparts (Sachs, D.H. (1994) *Veterinary Immunology & Immunopathology* **43**: 185-191). The breed of pigs described as miniature swine has a variety of advantages as a potential xenograft source. They achieve adult weights of approximately 100 - 150 kg, a size that is more compatible to human weights than that of the domestic pig, which reaches weights of over 500 kg. Through a selective breeding program over the past 20 years, partially inbred, miniature swine have been produced (Sachs et al. (1976) *Transplantation* **22**: 559-567; Sachs, D.H. 1992. In *Swine as models in biomedical research*, eds. M. Swindle, D. Moody, and L. Phillips, pp. 3-15. Ames Iowa State Univ. Press; Sachs, (1994) *Veterinary Immunology & Immunopathology* **43**: 185-191). This breeding program has resulted in herds of animals that are genetically well characterized and inbred at the major histocompatibility complex

(MHC). These animals have been used in large animal model studies for many years and have, like their domestic counterparts, very favorable breeding characteristics for being used as donors of organs in xenotransplantation.

The major barrier in the use of pig organs for transplantation has been the immune response to surface antigens present on pig cells. Thus, the most immediate problem in the xenografting of vascularized pig organs is that of hyperacute rejection (HAR) due to xenogeneic natural antibodies (XNAs) (Calne, R. 1970. *Transplantation Proc.* **2**: 550-556; Platt et al. (1991) *Transplantation* **52**:214-220). Surprisingly, the vast majority of human anti-pig XNAs are specific for a single carbohydrate linkage found on many glycoproteins on pig cells, galactose-α(1→3)-galactose (Gal-α(1→3)-Gal) (Sandrin et al. (1995) *Nature Medicine* **2**:1291-1267; Parker et al. 1994. *J. Immunology* 153: 3791-3803; Good et al. (1992) *Transplantation Proc.* **24**:559-562). Humans and Old World monkeys have lost the activity of the glycosyltransferase responsible for producing this structure and therefore are capable of producing antibodies specific for structures bearing the Gal-α(1→3)-galactose linkage (Galili and Swanson. (1991) *Proc Natl Acad Sci U S A.* **88**:7401-7404; Galili et al (1993) *Blood* **82**:2485-2493). The binding of XNAs to pig organs results in complement fixation and immediate destruction of the foreign graft. A variety of approaches have been taken to address the problem, including immunoaffinity adsorption of the XNAs, as well as genetic engineering of the pig.

However, even when HAR is prevented, there appears to be a secondary type of rejection related to antibody binding that has been described as delayed xenograft rejection or acute vascular rejection

(Blakely et al. (1994) *Transplantation* **58**:1059-1066; Leventhal et al. (1993) *Transplantation* **55**: 857-865; Platt. (1996) *Crit. Rev. Immunol.* 16: 331-358; Bach et al. (1996) *Immunology Today* **17**:379-384). The characteristics of this process include endothelial swelling, focal ischemia, inflammation and diffuse intravascular coagulation. The mechanisms involved in this process are controversial but appear to be antibody dependent and complement independent.

After the initial problem of XNAs is solved, however, the problem of a cellular response to the discordant xenograft must also be addressed (Fryer et al. (1994) *Transpl. Immunol.* **2**:87-93). Most recent evidence indicates that, at least in the case of pig-to-primate transplants, the T cell response is as great as or greater than that encountered for allotransplants (Murray et al. (1994) *Immunity* **1**:57-63; Yamada et al. (1995) *J. Immunology* **155**: 5249-5256). In addition, other cellular populations may also play a role in xenograft rejection (Blakeley et al. (1994) *Transplantation* **58**:1059-1066; Yamada et al. (1996) *Xenotransplantation* **3**: 179-183). It is also clear that there is a potent induced antibody response to xenografts that is dependent on T cell help, and that will therefore also require suppression of the cellular immune response (Tanaka et al. (1994) *Transplantation Proc.* **26**:1326-1327; Sachs et al. (1991) *Transplantation Proc.* **23**:28-31). For all of these reasons, it is likely that the amount of immunosuppression necessary to inhibit the cellular immune response in order to avoid rejection of xenografts will be greater than that required currently for control of allograft rejection. Because the current levels of immunosuppression used already place the transplant recipient on the borderline between rejection and drug-induced complications, it is highly desirable to eliminate as much as possible of the cellular response to xenografts through specific immunosuppression, i.e., through the induction of tolerance. The

induction of specific tolerance may be achieved through establishment of mixed hematopoietic chimerism, which results in specific elimination of the immune response to the transplant without diminishing immune responses to other antigens.

Tolerance to self major histocompatibility (MHC) antigens occurs during T cell maturation in the thymus so that exposure of the immune system to MHC antigens during ontogeny can cause the immune system to lose reactivity to those antigens, thus leaving the animal specifically tolerant towards its own antigens. It would therefore be highly advantageous for efforts at using xenotransplantation if such otherwise natural prenatal processes could be adapted for use in adult recipients of xenografts. The present invention solves this problem by providing a means of inducing tolerance (i.e., reducing the severity and/or eliminating the immunological response to the transplant), especially to porcine tissue, using hematopoietic stem cells, especially porcine hematopoietic stem cells (pHSCs).

BRIEF SUMMARY OF THE INVENTION

The present invention relates to a means of inducing tolerance to cells, tissues, and organs of a xenogeneic source by transplanting pHSCs that subsequently engraft in the recipient bone marrow and proliferate, differentiate, and function immunologically as self cells in the recipient.

It is one object of the present invention to provide a process for enhancing engraftment of xenogeneic hematopoietic cells in a primate

receiving xenogeneic bone marrow-derived cells, comprising administering to said primate, separately or as a mixture, of an effective amount of a transforming growth factor beta (TGF-β) antagonist and an effective amount of a tumor necrosis factor alpha (TNF-α) antagonist. Such administration may or may not follow administration of a conditioning regimen, which conditioning regimen may be a myeloablative regimen.

In one embodiment, the present invention relates to the just described process wherein the bone-marrow-derived cells are hematopoietic progenitor cells. In another embodiment these cells may be peripheral blood stem cells.

It is also an object of the present invention to provide a process for enhancing engraftment of xenogeneic hematopoietic cells in a primate receiving xenogeneic bone marrow-derived cells wherein such process contains a conditioning regimen, such regimen being either a myeloablative regimen or a non-myeloablative regimen.

The process of claim 4 wherein said myeloablative regimen comprises removal from said primate of a sample of bone marrow-derived cells followed by *in vitro* depletion of either $CD2^+$ cells or both $CD2^+$ cells and $CD20^+$ cells from said sample, and subsequent reintroduction of said bone marrow cells back into said primate. Such process will also commonly employ whole body irradiation to destroy all remaining hematopoietic stem cells.

Another embodiment of the present invention employs a non-myeloablative conditioning process selected from the group consisting of whole body irradiation, commonly using a non-lethal dose, administration of horse anti-human thymocyte globulin (ATG), thymic irradiation, splenectomy, anti-T cell antibodies as well as co-stimulatory

blockade molecules such as anti-CD40 Ligand antibody and CTLA4Ig and any combination of the foregoing.

In a specific embodiment, the present invention relates to a process wherein said xenogeneic bone marrow-derived cells are hematopoietic progenitor cells.

In another specific embodiment, the methods of the present invention employ xenogeneic bone marrow-derived cells that are porcine cells.

Another embodiment of the present invention may also employ cytokines that are porcine cytokines. In a more specific embodiment, these cytokines may be selected from the group consisting of interleulin-3 (IL-3), stem cell factor (SCF), and granulocyte-monocyte-colony stimulating factor (GM-CSF).

In one embodiment, the primate used in the present invention is a human.

The present invention also provides methods for enhancing engraftment that employ a specific TGF-β antagonist selected from the group consisting of anti-TGF-β antibodies, soluble TGF-β receptors, latency-associated peptide (LAP) and lysofilline, including active fragments thereof.

The present invention also provides methods for enhancing engraftment that employ a specific TNF-α antagonist selected from the group consisting of anti-TNF-α antibodies and soluble TNF-α receptors, including active fragments thereof.

In a most preferred embodiment, the present invention utilizes a mixture of antagonists that comprises an anti-TGF-β antibody, or an active fragment thereof, and an anti-TNF-α antibody, or an active fragment thereof.

In this embodiment, both antibodies are monoclonal antibodies, or active fragments thereof.

It is also an object of the present invention to provide a composition comprising a therapeutically effective amount of a transforming growth factor beta (TGF-β) antagonist and a tumor necrosis factor alpha (TNF-α) antagonist, especially where these antagonists are antibodies.

BRIEF DESCRIPTION OF THE FIGURES

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 is illustrative of the growth of porcine or primate (cynomolgus monkey) BMC (bone marrow cell) cultures on a primate stroma in the presence of pSCF (porcine stem cell factor) and pIL-3 (porcine interleukin-3) over a period of four weeks. (a) Total cell number per well, (b) total colony forming units per well. The space indicates a change in scale with 350 being the end of the first scale and the start of the second scale. Error bars represent ± 1 SEM (standard error of the mean) for six (6) separate experiments. Results in Figure 1 show that porcine hematopoiesis is not maintained to the same extent as primate hematopoiesis on a primate stroma.

Figure 2 illustrates the growth of porcine and primate BMCs cultured together on a primate stroma in the presence of pSCF and pIL-3 over a period of four weeks. At each week the pig and primate cells were separated from the co-cultures to determine contributions from each BMC population. (a) Total cell number per well, (b) total colony forming units (CFU) per well. Error bars represent ± 1 SEM from six separate experiments. Figure 2 shows porcine and primate cell number and CFU content after separation from mixed co-cultures using immunomagnetic selection with species specific antibodies.

Figure 3 depicts the growth of porcine BMCs grown in Transwell® inserts above no stroma, porcine stroma and primate stroma

alone. All cultures were grown in the presence of pSCF and pIL-3. (a) Total cell number per well, (b) total colony forming units per well. Error bars represent ± 1 SEM for three separate experiments. As demonstrated in Figure 3, porcine progenitors were best maintained and expanded when cultured above porcine stroma, indicating that cells in the lower chamber can influence cell growth in the upper chamber, presumably mediated by soluble factors.

Figure 4 depicts the growth of porcine BMCs grown in Transwell® inserts above: primate stroma alone, porcine BMCs on primate stroma, primate BMCs on primate stroma and porcine BMCs and primate BMCs on primate stroma. All cultures were grown in the presence of pSCF and pIL-3. (a) Total cell number per well, (b) total colony forming units per well. Error bars represent ± 1 SEM for three separate experiments. Figure 4 shows that when additional BMCs (porcine, primate or both) were added to primate stroma in the lower chambers, there was a further marked decline in total cell and CFU numbers as compared to primate stroma alone.

Figure 5 depicts the growth of porcine BMCs grown on primate stroma in the presence of porcine SCF and porcine IL-3 with no antibody; with blocking antibody to TGF-β; with blocking antibody to TNF-α; with blocking antibodies to TGF-β and TNF-α. (a) Total cell number per well, (b) total colony forming units per well. Error bars represent ± 1 SEM for five separate experiments. The addition of antibodies to both TGF-β and TNF-α produced the most striking effect as a marked enhancement of cell and CFU output was seen over the first three weeks of culture.

Figure 6 depicts the growth of primate BMCs grown on primate stroma in the presence of hSCF (human stem cell factor) and hIL-3 (human interleukin-3) with no antibody; with blocking antibody to TGF-β; with blocking antibody to TNF-α; with blocking antibodies to TGF-β and TNF-α. a) Total cell number per well b) Total colony forming units per well. The addition of the two antibodies did not appear to improve the enhancement of cell number and CFU output.

Figure 7 depicts the growth of porcine BMCs grown on porcine stroma in the presence of pSCF and pIL-3 with no antibody; with blocking antibody to TGF-β; with blocking antibody to TNF-α; with blocking antibodies to TGF-β and TNF-α. a) Total cell number per well b) Total colony forming units per well. The addition of the blocking antibodies resulted in a relatively small effect on cell and CFU output.

Figure 8 depicts the growth of porcine and primate allogeneic cultures with species specific SCF and IL-3. The figure depicts results for porcine cells with no inhibitory cytokine, or with 1ng/ml huTGF-β, or with 10ng/ml huTGFβ; primate cells with no inhibitory cytokine, or with 1ng/ml huTGF-β, or with 10ng/ml huTGF-β. a) Cells per well as % of controls b) Total colony forming units per well as % of controls. These results demonstrate that porcine BMCs are inhibited by huTGF-β to a much greater extent than primate cells are. (m indicates monkey, p indicates porcine).

Figure 9 illustrates the growth of porcine and primate allogeneic cultures with species specific SCF and IL-3: porcine cells with no inhibitory cytokine; with 1ng/ml pTGF-β; and with 10ng/ml pTGF-β; primate cells with no inhibitory cytokine; with 1ng/ml pTGF-β; and with 10ng/ml pTGF-β. a) Cells per well as % of controls b) Total colony forming units per well as % of controls. The results show that porcine

BMCs are more sensitive than primate BMCs to porcine TGF-β. (m indicates monkey, p indicates porcine).

Figure 10 illustrates the growth of porcine and primate allogeneic cultures with species specific SCF and IL-3: porcine cells with no inhibitory cytokine; with 1ng/ml huTNF-α; and with 10ng/ml huTNF-α; primate cells with no inhibitory cytokine; with 1ng/ml huTNF-α; and with 10ng/ml huTNF-α. a) Cells per well as % of controls b) Total colony forming units per well as % of controls. The porcine cells were inhibited to a much greater extent than primate cells, similar to the results with TGF-β. (m indicates monkey, p indicates porcine).

Figure 11 illustrates the growth of porcine and primate allogeneic cultures with species specific SCF and IL-3: porcine cells with no inhibitory cytokine; with 1ng/ml pTNF-α; and with 10ng/ml pTNF-α; primate with no inhibitory cytokine; with 1ng/ml pTNF-α; and with 10ng/ml pTNF-α. a) Cells per well as % of controls b) Total colony forming units per well as % of controls. The results obtained from primate cultures show that porcine TNF-α appeared to be stimulatory, resulting in 2-fold increases in CFU production at 3 to 6 weeks. Porcine cells were inhibited by pTNFα. (m indicates monkey, p indicates porcine).

Figure 12 demonstrates that addition of either Latency Associated Peptide (LAP) or soluble TGF receptor II (sTGFrII) results in a similar improvement of the growth of porcine BMCs as achieved using the anti-TGF antibody.

Figure 13 shows that when the addition of soluble TNFrI and II is compared to the use of anti-TNF-α antibody the results are equivalent.

Figure 14 shows that the addition of LAP to anti-TNF-α antibody has equivalent results as compared to the anti-TGF-β/anti-TNF-α antibodies combination.

DETAILED DESCRIPTION OF THE INVENTION

It has been known for many years that production of fully allogeneic chimeras through bone marrow transplantation carries with it the induction of transplantation tolerance to other tissues and organs from the donor of the allogeneic none marrow stem cells (Rapaport et al. (1978) *J. Clin. Invest.* **61**: 790-800). However, there are two major problems with this procedure as a means of inducing tolerance: (1) if mature T cells are not removed from the allogeneic bone marrow, then graft-versus-host disease (GVHD) may result; and (2) if mature T cells are removed from the allogeneic bone marrow inoculum, then the chimeras that result are relatively immunoincompetent. As such it is preferable that a state of mixed hematopoietic chimerism be developed in which both donor and recipient hematopoietic stem cells exist in the recipient, thereby allowing the recipient to reestablish immunocompetence, which retaining the ability to recognize the donor antigens as self.

The present invention relates to methods of increasing the extent of mixed hematopoietic chimerism as a means of inducing tolerance to xenogeneic organs, tissues and cells, especially following transplant. Induction of such tolerance has been found to be facilitated by the

addition of porcine cytokines, which have an effect on pHSC engraftment and enhances the level of engraftment [See: Giovino et al. (1997) *Xenotransplantation* **4**: 112-119; Hawley et al. US Patent Nos. 5,589,582, 5,858,963, and 5,863,528], the entire contents of which are incorporated herein by reference). It is also necessary to create niches for the progenitor stem cells to engraft. Methods for creating niches include the use of irradiation or myeloablative drugs. [See: Sachs US patent 5,876,708; Sykes WO 97/41863; Sykes WO, 99/39726; Sykes and Sayegh, WO 99/39727, the contents of which are incorporated entirely herein by reference].

In accordance with the present invention, it has further been discovered that inhibitory factors that influence the growth of pHSCs (porcine hematopoietic stem cells) are present in the primate bone marrow microenvironment. The effects of such inhibitory factors may be overcome by the addition/administration of reagents that inhibit or otherwise eliminate or reduce the activity of the inhibitors. Specifically, when anti-transforming growth factor-beta (anti-TGF-β) and anti-tumor necrosis factor alpha (anti-TNF-α) antibodies are present in *in vitro* cultures of pHSCs being cultured on primate stroma the total cell and colony forming unit numbers (CFUs) are increased.

Hematopoiesis is known to be regulated by a complex interaction of bone marrow cells, stromal cells and growth factors. Hematopoietic lineage restricted stem cell growth has been shown to be inhibited by the addition of exogenous TGF-β to long-term bone marrow cultures (LTBMC) Van Ranst et al. 1996. *Experimental Hematology* 24: 1059-1515. Treatment of such cultures with anti-TGF-β antibody has resulted in a five-to-twenty fold increase in non-adherent cells in the cultures by week 4 when compared to untreated controls (Waegell et

al. 1994. *Experimental Hematology* 22: 1051-1057). TNF-α, a monocyte/macrophage product, is another such mediator of hematopoiesis with a wide range of biological activities. In the LTBMC TNF-α has been shown to suppress formation of relatively mature progenitors as well as more primitive high proliferative potential colony forming cells (HPPCFCs) (Rogers and Berman (1994) *J. Immunology* **153**:4694-4703).

In accordance with the present invention, it has been discovered that the addition of both anti-TNF-α and anti-TGF-β antibodies together to *in vitro* cultures of porcine stem cells and primate stroma leads to a more than additive effect (i.e., a synergistic effect).

Rubin et al (EPO Patent Publication 0218868, April 22 1987) discloses murine monoclonal antibodies to human TNF. Other investigators have described rodent or murine antibodies to recombinant human TNF which had neutralizing activity *in vitro* (Lian et al. (1986) *Biochem, Biophys. Res Comm.* **137**:847-854; Meager et al. (1987) *Hybridoma* **6**: 305-311; Fendly et al. (1987) *Hybridoma* **6**: 359-369; Bringman et al. (1987) *Hybridoma* **6**: 489-507; Hirai et al. (1987) *J.Immunol. Methods* **96**: 57-62; Jumming et al US Patent 5,919,452).

Anti-human TNF-α antibodies are commercially available, e.g. from R & D Systems, Minneapolis, MN).

Anti-human TGF-β antibodies are also well described in the literature and are commercially available, e.g. from R&D Systems, Minneapolis, MN.

As used herein, the term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (MAbs), chimeric antibodies, humanized antibodies, and anti-idiotypic (anti-Id) antibodies, as well as fragments, regions or derivatives thereof, including Fab and $F(ab')_2$, and provided by any known technique, such as, but not limited to, enzymatic cleavage, peptide synthesis or recombinant techniques. Such anti-TNF-α and anti-TGF-β antibodies of the present invention are capable of binding portions of TNF-α and TGF-β, respectively, that inhibit the binding of TNF-α (or TGF-β) to TNF-α (or TGF-β) receptors.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein. (1975) *Nature* **256**:495-497; U.S. Pat. No. 4,376,110; Ausubel et al, eds., (1987) 1992. *Current Protocols In Molecular Biology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., and Harlow and Lane. (1988) *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory; Colligan et al. 1992, 1993. eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., the contents of which references are incorporated entirely herein by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and any subclass thereof. A hybridoma producing a MAb of the present invention may be cultivated *in vitro*, *in situ* or *in vivo*.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable

regions derived from a murine MAb and a human immunoglobulin constant region, which are primarily used to reduce immunogenicity in application and to increase yields in production, for example, where murine MAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric MAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al. (1984) *Proc. Natl. Acad. Sci. USA* **81**:3273-3277; Morrison et al. (1984) *Proc. Natl. Acad. Sci. USA* **81**:6851-6855; Boulianne et al. (1984) *Nature* **312**:643-646; Cabilly et al., European Patent Application 125023 (published Nov. 14, 1984); Neuberger et al. (1985) *Nature* **314**:268-270; Taniguchi et al., European Patent Application 171496 (published Feb. 19, 1985); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Neuberger et al., PCT Application WO 86/01533, (published Mar. 13, 1986); Kudo et al., European Patent Application 184187 (published Jun. 11, 1986); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Sahagan et al. 1986. J. Immunol. **137**:1066-1074; Robinson et al., International Patent Publication #PCT/US86/02269 (published May 7, 1987); Liu et al. (1987) *Proc. Natl. Acad. Sci. USA* **84**:3439-3443; Sun et al. (1987) *Proc. Natl. Acad. Sci. USA* **84**:214-218; Better et al. (1988) *Science* **240**:1041-1043; and Harlow and Lane. (1988) *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory). Humanized antibodies are also well described in the art, e.g. Queen US patent 5,693,762. These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the MAb with the MAb to which an anti-

Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Pat. No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original MAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a MAb, it is possible to identify other clones expressing antibodies of identical specificity.

The antibodies of the present invention can include at least one of a heavy chain constant region, a heavy chain variable region, a light chain variable region and a light chain constant region, wherein a polyclonal Ab, monoclonal Ab, fragment and/or regions thereof include at least one heavy chain variable region or light chain variable region which binds a portion of TNF-α (or TGF-β) and inhibits and/or neutralizes at least one TNF-α (or TGF-β) biological activity.

Preferred antibodies of the present invention are high affinity human-murine chimeric anti-TNF-α (or TGF-β) antibodies, and fragments or regions thereof, that have potent inhibiting and/or neutralizing activity *in vivo* against human TNF-α (or TGF-β).

The immunoglobulin gene can be from any vertebrate source, such as murine, but preferably, it encodes an immunoglobulin having a substantial number of sequences that are of the same origin as the

eventual recipient of the immunoreceptor peptide. For example, if a human is treated with a molecule of the invention, preferably the immunoglobulin is of human origin.

TNF-α (or TGF-β) receptor constructs for linking to the heavy chain can be synthesized, for example, using DNA encoding amino acids present in the cellular domain of the receptor. Thus, one skilled in the art, once armed with the present disclosure, would be able to create TNF-α (or TGF-β) receptor fusion proteins using portions of the receptor that are known to bind TNF-α (or TGF-β).

Anti-TNF-α (or TGF-β) peptides and/or MAbs of the present invention can be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier, diluent or excipient selected on the basis of the chosen route of administration and standard pharmaceutical practice. The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1.0 to 5, and preferably 1 to 10 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

As a non-limiting example, administration can be provided as a daily dosage of anti-TNF-α peptides, monoclonal chimeric and/or murine antibodies of the present invention 0.1 to 100 mg/kg, per day, for at

least one day, or alternatively, at least one week, or any combination thereof, using single or divided doses of every 24, 12, 8, 6, 4, or 2 hours, or any combination thereof.

Since circulating concentrations of TNF-α tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome (Hammerle, A. F. et al., 1989,) or can only be detectable at sites of TNF-α-mediated pathology, it is preferred to use high affinity and/or potent in vivo TNF-α -inhibiting and/or neutralizing antibodies, fragments or regions thereof, for both TNF-α immunoassays and therapy of TNF-α-mediated pathology. Such antibodies, fragments, or regions, will preferably have an affinity for hTNF-α, expressed as K_a, of at least 10^8 M^{-1}, more preferably, at least 10^9 M^{-1}, most preferably 10^{10} M^{-1}.

The circulating concentrations of TGF-β are in the range of 1-50 ng/ml (Wakefield et al. (1995) *Clinical Cancer Res.* **1**:129-136; Kyrtsonis et al. (1998) *Med Oncol.* **15**: 124-128).

Dosage forms (composition) suitable for internal administration generally contain from about 0.1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

For parenteral administration, anti-TNF-α (or anti-TGF-β) peptides or antibodies can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline,

Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

The current invention pertains to the discovery that combination therapy, involving the use of a TGF-β antagonist together with a TNF-α antagonist produces markedly superior results versus use of each agent alone in the establishment of mixed hematopoietic chimerism, for example, in pig-to-baboon xenografts. TGF-β antagonists include agents which block, diminish, inhibit, or interfere with TGF-β activity and may include anti-TGF-β antibodies, soluble TGF-β receptors, or small molecule inhibitors such as lysofylline. TNF-α antagonists include agents which block, diminish, inhibit, or interfere with TNF-α activity and may include anti-TNF-α antibodies, soluble TNF-α receptors, or small molecule inhibitors such as lysofylline.

In one embodiment of the current invention, anti-TNF-α and anti-TGF-β antibodies are administered together. In another embodiment of the invention, anti-TNF-α antibody is administered in conjunction with a anti-TGF-β agent other than an antibody. In another embodiment of the invention anti-TGF-β antibody is administered in conjunction with an anti-TNF-α agent other than an antibody. In a further embodiment TGF-β and TNF–α antagonists other than antibodies are administered.

TGF-β is secreted in the form of an inactive complex non-covalently linked to latency associated peptide (LAP). Once the two components are cleaved TGF-β becomes active (Ribeiro SM, (1999) J.

Biol. Chem. **274** 13586-13593). LAP has been shown to inhibit the activity of TGF-β (Bottinger et al. (1996) *Proc. Natl. Acad. Sci.USA*. **93**: 5877-5882). For this reason it is feasible to substitute the addition of anti-TGF-β antibody with LAP.

Soluble TNF receptors have been shown to block the activity of TNF-α, either occurring naturally *in vivo* (Aderka et al. (1992) *J.Exp.Med.* **175**: 323-329: Pinckard et al. (1997) *J. Immunology* **158**: 3869-3873) or by the addition of soluble receptors to block effects of TNF-α (Eliaz et al. 1996. *Cytokine* **8**:482-487; Camussi and Lupia. (1998) *Drugs* **55**: 613-620). Since the soluble TNF receptors can inhibit TNF activity it is reasonable to consider their use within our system.

Soluble TGF-β receptor II has been shown to inhibit the activities of TGF-β (Smith et al. (1999) *Circ. Res.* **84**:1212-1222; Komesli et al. (1998) *Eur. J. Biochem*. **254**: 505-513). These results suggest that sTGFrII could be used to substitute for TGF-β antibody.

In keeping with the foregoing disclosure, the present invention relates to a process for enhancing engraftment of xenogeneic hematopoietic cells in a primate receiving xenogeneic bone marrow-derived cells, comprising administering to said primate, separately or as a mixture, of an effective amount of a transforming growth factor beta (TGF-β) antagonist and an effective amount of a tumor necrosis factor alpha (TNF-α) antagonist.

Such administration may or may not follow administration of a conditioning regimen. Thus, in a particular aspect, the present invention

includes a process for enhancing engraftment of xenogeneic hematopoietic cells in a primate receiving xenogeneic bone marrow-derived cells, comprising:

(a) administering to said primate a conditioning regimen;

(b) administration of a sample of xenogeneic bone marrow-derived cells; and

(c) administering to said primate, separately or as a mixture, of an effective amount of a transforming growth factor beta (TGF-β) antagonist and an effective amount of a tumor necrosis factor alpha (TNF-α) antagonist.

In accordance with the disclosure herein, and whether or not a conditioning regimen is used, the xenogeneic cells may be bone marrow cells or bone-marrow-derived cells, including peripheral blood stem cells, and preferably hematopoietic cells, most preferably hematopoietic progenitor cells.

In accordance with the present invention, there is optional administration before, during or after step (b), of an effective amount of at least one cytokine derived from the donor species. In accordance with step (c), there is administration to said primate, separately or as a mixture, of an effective amount of a transforming growth factor beta (TGF-β) antagonist and an effective amount of a tumor necrosis factor alpha (TNF-α) antagonist, the latter two substances being administered either separately or as a mixture. As a general rule, the sequence and timing of each and all of the steps disclosed according to the methods of the present invention are to be left to the sound discretion of the researcher and/or clinician, as determined by the requirements and exigencies of the overall procedure being conducted, with the sequence of steps as disclosed herein merely constituting one embodiment of the invention.

In keeping with the disclosure herein, the conditioning regimen may be either a myeloablative regimen or a non-myeloablative regimen. Where said regimen is myeloablative, the method comprises complete elimination of the recipient's hematopoietic progenitor cells such that without the administration of exogenous cells (in accordance with the present invention this would comprise autologous and xenogeneic cells) the patient would die. Such elimination is generally achieved through lethal dose of whole body irradiation. The method of the present invention therefore comprises prior removal from said primate, especially a human patient, of a sample of cells, such as bone marrow cells or bone marrow-derived cells, especially autologous hematopoietic cells, followed by, preferably, *in vitro* depletion of either $CD2^+$ cells or, preferably, both $CD2^+$ cells and $CD20^+$ cells from said sample, and subsequent reintroduction of said cells back into said primate, this latter step being carried out at some point in the overall procedure, along with the sample of xenogeneic cells, with the exact timing left to the sound discretion of the researcher and/or clinician conducting such procedure.

Where the method of the invention is to be carried out using a non-myeloablative conditioning regimen, such regimen may comprise, but is not limited to, whole body irradiation, administration of horse anti-human thymocyte globulin (ATG), thymic irradiation, splenectomy, and/or any combination of the foregoing. Preferably, such non-myeloablative conditioning regimen involves partial elimination of the recipient's (i.e., the primate's) hematopoietic progenitor cells through the use of non-lethal doses of radiation, or anti-T cell antibody, or chemicals such as cyclophosphamide. Further in keeping with the invention disclosed herein, such non-myeloablative steps may be conducted in combination and in any temporal sequence, as dictated by the needs and exigencies of the overall procedure being conducted and

as determined by the researcher and/or clinician conducting said procedure. Further in keeping with the invention as disclosed herein, the conditioning regimen may be either a myeloablative or a non-myeloablative procedure, as dictated by the needs of the procedure being carried out. Once again, the timing and use of such steps, and the sequence thereof, is left to the sound discretion of the researcher and/or clinician conducting such procedures.

In accordance with the present invention, the xenogeneic bone marrow cells introduced into the recipient animal, or primate, may be any type of bone marrow-derived cells or hematopoietic progenitor cells as required by the procedure being conducted and as dictated by the needs and preferences of the researcher and/or clinician.

In a preferred embodiment, the xenogeneic bone marrow cells introduced into the recipient animal as part of the present invention are hematopoietic stem cells, most preferably porcine hematopoietic stem cells.

The process of the present invention also optionally provides for the administration to the recipient animal of a sample of a cytokine, preferably a mixture of cytokines, and most preferably a mixture of cytokines derived from the same species as the xenogeneic donor organism, the latter preferably being a pig. Thus, where the donor animal is a pig, the xenogeneic bone marrow cells or bone marrow-derived cells, such as hematopoietic progenitor cells, and cytokines are both porcine in origin.

In a most preferred embodiment, the cytokines are selected from the group consisting of interleulin-3 (IL-3), stem cell factor (SCF), and granulocyte-monocyte-colony stimulating factor (GM-CSF).

In accordance with the methods of the present invention, the recipient animal, such as a human or other primate, will also receive a dosage of substances comprising TGF-β and TNF-α antagonists. In a preferred embodiment, such TGF-β antagonist is selected from the group consisting of anti-TGF-β antibodies, soluble TGF-β receptors, and latency-associated peptide (LAP) including active fragments thereof and said TNF-α antagonist is selected from the group consisting of anti-TNF-α antibodies and soluble TNF-α receptors, including active fragments thereof. In a most preferred embodiment, the TGF-β antagonist is an anti-TGF-β antibody, or an active fragment thereof, and the TNF-α antagonist is an anti-TNF-α antibody, or an active fragment thereof. Such active fragments can include, but are not limited to, Fab and $F(ab')_2$ fragments which commonly retain the antigen specificity and affinity of the complete antibody molecule. Such active fragments can also include heavy-light chain dimers as well as dimers made up solely of the variable regions of the antibodies. Antibodies useful for the present invention also include bispecific antibodies. Such antibodies may be polyclonal or monoclonal and can include chimeric antibodies, humanized antibodies and other types of altered antibody molecules. Antibodies useful in practicing the present invention include recombinant as well as plasma derived antibodies. Where such antibodies are recombinant in nature, these may be produced in cells of any type that can synthesize and secrete antibody molecules, including mammalian cells in culture, especially myeloid and Chinese hamster ovary (CHO) cells, as well as plant cells. The polypeptides making up the antibodies useful for practicing the present invention may also be synthesized by chemical means without the need for intermediary cellular production.

The present invention also relates to processes for enhancing the growth rate of bone marrow cells *in vitro* comprising growing said cells

on a stromal layer in a culture medium comprising species specific cytokines (i.e., cytokines derived from the same species as the bone marrow cells) and further comprising a growth-stimulating amount of a transforming growth factor beta (TGF-β) antagonist and a growth-stimulating amount of a tumor necrosis factor alpha (TNF-α) antagonist.

In a preferred embodiment, the TGF-β antagonist is selected from the group consisting of anti-TGF-β antibodies, soluble TGF-β receptors, latency-associated peptide (LAP) and lysofilline, and active fragments thereof.

In a preferred embodiment, the TNF-α antagonist is selected from the group consisting of anti-TNF-α antibodies and soluble TNF-α receptors, and active fragments thereof.

In a preferred embodiment, the bone marrow cells are hematopoietic stem cells, most preferably porcine hematopoietic stem cells. In a separate preferred embodiment, the stromal cells are either porcine stromal cells or primate stromal cells and, in a most preferred embodiment, the bone marrow cells are porcine hematopoietic stem cells.

In a most preferred embodiment, the primate is selected from the group consisting of baboons, cynomolgus monkeys and humans.

In accordance with the methods of the present invention, the enhanced growth rate is measured as either an increase in the total number of cells or an increase in the number of colony forming units, with the latter being preferred. Said enhanced growth rate may also

comprise an increase in both total cell number and colony forming units.

In utilizing the methods disclosed herein, the amounts of any and all substances employed either for inducing immunological tolerance or enhancing cell growth rate are understood to be "effective amounts" of said substances and additives. As used herein, the term "effective amount" means at least the minimum amount of the substance required to produce the desired biological effect and is in no way limited to the particular amounts and dosages recited in the following examples. Thus, the "effective amount" of any such substance or additive may need to be determined for the particular experiment or clinical procedure being carried out and may also depend on the identity of the recipient and donor species as well as the species from which the particular substance or additive is derived. Exact dosages are therefore left to the sound discretion, based on the sound experience and knowledge of the prevailing art, of the researcher and/or clinician conducting the procedure.

As an example doses of antibodies should not be less than 10 μg/kg/day, which would constitute a minimum effective amount.

The present invention will now be further described by way of the following non-limiting examples. In applying the disclosure of these examples, it should be kept clearly in mind that other and different embodiments of the methods disclosed according to the present invention will no doubt suggest themselves to those of skill in the relevant art.

EXAMPLE 1

Porcine Hematopoiesis in the Primate Microenvironment - *in vitro* studies

Materials and Methods

Animals

Pig femurs and humeri were collected from a herd of MHC class I and II inbred miniature swine (Sachs et al. (1976) *Transplantation*: **22**:559). Primate femurs were obtained from cynomolgus monkeys, *Macaca fascularis* (Covance, Alice, TX).

Enrichment of pig bone marrow cells

Porcine bone marrow cells were harvested from the femurs and humeri by aseptically scraping with bone curettes and flushing the cavity with Dulbecco's phosphate buffered saline without calcium and magnesium (D-PBS, Gibco, Grand Island, NY) containing 5% citrate-phosphate-dextrose solution (Sigma, St. Louis, MO) and 20 μg/ml gentamycin (Gibco). Cells were washed 2 times at 2000 rpm for 5 minutes in D-PBS, resuspended in D-PBS then layered over Histopaque (sp.gr. 1.077; Sigma) and centrifuged at 400 x g for 25 minutes. Low density cells were collected, washed and resuspended in Iscove's Modified Dulbecco's Media (IMDM, Gibco) containing 10% fetal bovine serum (FBS) and 50 U/ml DNase (Sigma). The cells were placed in tissue culture flasks (Costar, T150) at 5 x 10^6/ml and allowed to adhere for 60 minutes at 37° C, 5% CO_2 and 95% humidity.

For isolation of stromal cell and hematopoietic progenitor cell enriched bone marrow fractions, the non-adherent cells were loaded directly into a sterilized elutriator system (Beckman Instruments, Palo Alto, CA) using a Beckman JE-6B rotor system equipped with a 40 ml chamber. Elutriation was performed using a constant rotor speed of 2040 rpm and cells were separated by increasing the flow rate. Cells (1-9 x 10^9) were loaded at a flow rate of 45 ml/min. After all the cells had entered into the chamber, the media flow rate was increased to 50 ml/min and a first fraction (700 ml) was collected. Fraction 2 (700 ml) was collected by increasing the flow rate to 75 ml/min. After collecting the second fraction, media flow and rotor were turned off and chamber contents (fraction 3) were harvested aseptically under a biological hood. Consistent with previous reports (Monroy et al. 1984. *Exp Hematol*: 12:384; Wagner et al. 1988. *Blood* 72:1168) the 75 ml/min fraction was enriched for CFU-Mix and BFU-E bone marrow progenitors.

This population was further enriched by depleting T-cell and myeloid populations utilizing porcine specific anti-CD2 (MSA-4) and anti-myeloid(74-22-15A) monoclonal antibodies (provided by David H. Sachs, Massachusetts General Hospital, Charlestown, MA). Antibodies were added at a concentration of 15μg/1x10^9 cells in 5 mls of D-PBS-with 0.5% BSA for 30 minutes at 4° C. Cells were then centrifuged for 5 min at 2000 rpm, resuspended in 10 ml of PBS + BSA and washed again. Following the wash cells were then incubated with Dynal M450 magnetic beads according to the manufacturer's instructions. The unbound cells were used in these experiments as a population enriched for porcine progenitor cells.

Primate BMC and enrichment for $CD34^+$ cells

Bone marrow cells from cynomolgus monkeys harvested from the femurs of donor animals, washed in D-PBS, and low density cells were isolated by separation over Histopaque. The low density cells were depleted of adherent cells by culturing in T150 flasks for an hour at 37°C, 5% CO_2, in IMDM, 10% FBS. Non-adherent cells were used for either formation of primary stromal cell cultures or isolation of $CD34^+$ cells. The $CD34^+$ cells were isolated on antibody affinity columns (CellPro, Bothell, WA) according to the manufacturer's instructions.

Long term bone marrow cultures

Stromal cultures

Preformed primary porcine stromal cell cultures were set up using fraction 3 of elutriated BMC. Then 1 x 10^6 cells/well were plated into 24 well plates coated with 1% gelatin (Sigma) in 1 ml of LTBMC medium (M-199 base medium (Gibco), 10% FBS, 10% horse serum (Gibco), 10^{-6}M hydrocortisone (Sigma)). Cultures were incubated at 37°C, 5% CO_2 and 95% humidity. Stromal cell cultures from primate bone marrow were established and treated similarly except that 2 x 10^6 cells/well were plated and the cultures were initially grown at 37°C for the first week and at 33°C for subsequent weeks. Media were demi-depleted and supplemented with fresh media at weekly intervals until a confluent adherent cell layer was present (~3 weeks). One day prior to use, stromal cell cultures were irradiated with a dose of 10 Gy using a cesium irradiator (J.L.Shephard, San Fernando, CA).

Porcine Cultures

The enriched pig BMC were seeded onto irradiated stromal cells at 2.5 x 10^5 cells/well in a 1 ml volume. Porcine growth factors (stem

cell factor (SCF), interleukin-3 (IL-3), GM-CSF) have been cloned (Hawley et al. 1997. *Xenotransplantation* 4:103-111) and expressed in *Pichia pastoris.* 2ng/ml of rpIL-3 (recombinant porcine interleukin-3) and 25ng/ml of rpSCF (recombinant porcine stem cell factor) were added to the LTBMC media at the initiation of the cultures and at each weekly feeding. Porcine cytokine concentrations used in this study were determined in titration experiments in long term bone marrow culture to provide the highest percentage of long term growth of pig cells (data not shown). Cultures were grown at 37°C, 5% CO_2, and 95% humidity. At weekly intervals media was demi-depleted and replenished with fresh media containing the appropriate growth factors. Adherent and non-adherent cells from 2 wells for each parameter were combined and enumerated at weekly time points. One aliquot from each harvest was assayed for CFU activity, and a second aliquot was used to assess cell morphology by microscopic examination after Diff-Quik (Baxter, MacGaw Park, IL) staining of cytospin preparations.

Primate Cultures

Primate $CD34^+$ cells were seeded onto primate stroma at $5x10^4$ cells per well per ml, also in pSCF+pIL-3. These cultures were set up as controls for the co-culture experiments where porcine and primate cells were cultured together on a primate stroma to evaluate survival of porcine cells in this mixed environment.

Mixed Co-cultures

Porcine enriched ($2.5x10^5$) and primate $CD34^+$ ($5x10^4$) BMCs were cultured together on a primate stroma in the presence of porcine cytokines to determine if porcine cells could survive in a primate hematopoietic microenvironment. These cell doses contained roughly equivalent number of pig and primate CFUs in the initial inoculum. To

determine survival of porcine cells within mixed co-cultures, cells were physically separated from each other. To separate porcine BMCs from primate BMCs after mixed co-culture, an anti-pan pig antibody was used. Cells were harvested and then resuspended in PBS + 0.5% BSA with 20% supernatant from an IgM anti-pan pig antibody H119 (kindly provided by David H. Sachs Massachusetts General Hospital, MA) at 100µl/1x10^5 cells and were incubated at 4°C for 30 minutes. Cells were then washed 1x then incubated with anti-mouse IgM microbeads (Miltenyi Biotech, Auburn , CA) according to the manufacturer's instructions. Cells were then passed over a magnetic column; bound cells (porcine population) and unbound cells (primate population) were then counted and placed into CFU assays. Preliminary experiments were conducted to test the effect of this separation protocol on fresh porcine BMCs and on porcine cells cultured on porcine stroma. Cell and CFU recovery was >95% of total input (data not shown).

Transwell® Cultures

To determine if soluble factors affected the growth of porcine cells, enriched porcine BMCs were plated on collagen coated Transwell® (Costar, Cambridge MA) inserts at 2.5x10^5 cells per insert above various parameters. Parameters below Transwell®s included: no stroma, porcine stroma alone, primate stroma alone, primate cells on primate stroma, porcine cells on primate stroma and primate and porcine cells on primate stroma. Cultures were fed weekly with pSCF and pIL-3, cells from the Transwell®s were counted and plated in CFU assays for determination of progenitor content.

Addition of Blocking Antibodies to Cultures

Antibodies to human TNF-α (Cat. No. AB-210-NA from R&D Systems, Minneapolis,MN), human TGF-β (Cat. No. AB-101-NA from

R&D Systems, Minneapolis,MN) and a combination of both were added weekly to the cultures at 25 μg/ml. Antibody titrations were carried out and 25ug/ml was found to be optimal for the maintenance of porcine cells (data not shown). Enriched porcine BMCs were plated either on porcine stroma (allogeneic cultures) or primate stroma (xenogeneic cultures) at $2.5x10^5$ cells/well in the presence or absence of pSCF + pIL-3. For primate allogeneic cultures, $5x10^4$ $CD34^+$ cells/well were plated on a primate stroma in the presence of either pSCF and pIL-3 or in the presence of hSCF and hIL-3.

Addition of Inhibitors to Cultures

Recombinant human and porcine TNF-α and TGF-β were added to cultures of porcine cells on porcine stroma and to primate cells on primate stroma.

In the porcine cultures, FICOLL™ separated BMCs were depleted of mature cells using anti-T-cell and myeloid antibodies. Cells were then plated at $2.5x10^5$ cells per well in the presence of pSCF (25ng/ml) and pIL-3 (2ng/ml) onto irradiated porcine stromal cells. Recombinant human TNF-α (R&D Systems), recombinant porcine TNFα (Endogen, Cambridge, MA), recombinant human TGF-β (R&D Systems) and recombinant porcine TGF-β (R&D Systems) were added to cultures at 1 and 10 ng/ml. At weekly intervals cells were harvested and placed into CFU assays for assessment of progenitors.

For primate allogeneic cultures, FICOLL™ separated primate BMCs were depleted of T and B cells by incubation with anti-CD2 and anti-CD20 antibodies (anti-Leu-5b and anti-Leu-16, Becton Dickinson, San Jose, CA) followed by depletion with mouse IgG magnetic beads

(Dynabeads M450, Dynal). Cells were plated at $2.5x10^5$ per well and cultured in 100 ng/ml of hSCF and hIL-3 in the presence or absence of human and porcine TNF-α and TGF-β.

Progenitor cell assays

Adherent and non-adherent cells were combined to assess progenitor cell content. Briefly, non-adherent cells were aspirated, wells were then rinsed with PBS and adherent cells were treated with trypsin EDTA (Gibco) for 2 minutes at 37°C and then pooled together. Progenitor cell content of porcine LTBMC was assayed by plating 25,000-100,000 cells in 1.1% methylcellulose (Stem Cell Technologies, Vancouver, Canada) supplemented with 30% FBS, porcine GM-CSF (5ng/ml), pIL-3 (2 ng/ml); pSCF (25 ng/ml), and 2 U/ml human Erythropoeitin (Epo) (R&D Systems). Colonies (>50 cells) were counted after 10-14 days in culture at 37°C, 5% CO_2. Each factor was individually titrated for its colony stimulating activity and concentrations that gave maximum stimulation were used in the assay. For the monkey CFU assay, the following combination of factors were added: hIL-3, 100 ng/ml; hIL-6 (Interleukin-6), 20 ng/ml; hSCF, 100 ng/ml; hEpo, 2 U/ml; hLIF (Leukemia Inhibitory Factor), 20 ng/ml; and hGM-CSF, 10 ng/ml (R&D Systems).

Results

Porcine Hematopoiesis in the Primate Microenvironment

Porcine and primate BMCs were cultured on a primate stroma for 4 weeks to examine the potential of primate stroma to support pig hematopoiesis as compared to its ability to support primate cells. All of the following cultures had pSCF and pIL-3 added. Maintenance of hematopoiesis was monitored in terms of cell number and CFU output in the cultures. Stem cell enriched porcine BMCs and primate $CD34^+$

cells were seeded into wells at cell numbers estimated to contain similar amounts of total CFU at the initiation of the cultures. Results in Figure 1 show that porcine hematopoiesis is not maintained to the same extent as primate hematopoiesis on a primate stroma. Throughout the 4 weeks of culture, total primate cell numbers were greater than the input whereas the total pig cell numbers were lower. There were fewer porcine CFUs than primate at each time point. In spite of being cultured in the presence of pig growth factors, porcine BMCs did not flourish as well as primate BMCs on a primate stroma. Previous studies have shown that maintenance of long-term hematopoiesis of porcine cells in these cultures occurs only in the presence of pig cytokines, although not to the same extent as when cultured on porcine stroma (Giovino et al. 1997. *Xenotransplantation* 4:112).

In order to simulate more closely the *in vivo* situation, primate $CD34^+$ BMCs were added to cultures of porcine BMCs on primate stroma (mixed co-cultures). Figure 2 shows porcine and primate cell number and CFU content after separation from mixed co-cultures using immunomagnetic selection with species specific antibodies. Cultures of primate or porcine cells alone on a primate stroma were also analyzed as a control for the separated cultures. A marked inhibition of porcine cell and CFU production in the mixed co-cultures was observed at all time points as compared to porcine cells alone on primate stroma. In contrast, little or no inhibition of primate cells occurred in these cultures. Thus porcine cell growth appears to be actively inhibited in the presence of primate cells. To determine if primate cell number was a factor in porcine cell inhibition cultures were set up with 2 doses of primate $CD34^+$ cells, one dose being 5 fold less than the dose used for experiments shown in Figure 2. The survival of porcine cells was comparable in mixed cocultures with either concentration of primate

cells (data not shown) indicating that the inhibitory effects of primate cells was similar over this cell dose range.

Effects of soluble factors from primate cultures on porcine BMCs-Transwell® cultures

To determine if soluble factors and/or cell contact were involved in the inhibition of pig progenitors, porcine BMCs were cultured in Transwell® inserts above various culture conditions. Throughout the culture period, cells in the upper chamber were evaluated for cell number and the presence of porcine hematopoietic progenitors. Culture systems utilized in the lower chambers were as follows: no stroma, primate stroma and porcine stroma. As demonstrated in Figure 3, porcine progenitors were best maintained and expanded when cultured above porcine stroma, indicating that cells in the lower chamber can influence cell growth in the upper chamber, presumably mediated by soluble factors. At weeks 2-5 above porcine stroma and weeks 4-6 above no stroma, the total number of colonies was greater than the initial CFU input. In contrast, when porcine BMCs were cultured above primate stroma, the total cell and colony numbers were much lower than those above porcine stroma or no stroma. These data show that the presence of primate stroma in the lower chamber can inhibit the growth of porcine BMCs. By the second week porcine BMCs in Transwell®s form their own stroma. The contribution of autologous stroma is important in maintaining porcine progenitors and in regulating porcine hematopoiesis. With regards to no stroma, at the first half of the experiment CFUs are lower than those over porcine stroma, at later weeks CFUs increase, this is probably due to the formation of porcine stroma within the Transwell® to support growth.

To evaluate the effects of additional culture combinations in the lower chambers, porcine cells were cultured in the upper chambers above the following: $CD34^+$ primate BMCs on primate stroma, porcine

BMCs on primate stroma and both porcine and primate BMCs on primate stroma. Figure 4 shows that when additional BMCs (porcine, primate or both) were added to primate stroma in the lower chambers, there was a further marked decline in total cell and CFU numbers as compared to primate stroma alone. From these results, it appears that soluble factors released from the primate stroma are inhibitory to porcine BMCs and that this effect is enhanced by the addition of either porcine or primate BMCs to the primate stroma.

Addition of Blocking Antibodies to TGF-β and TNF-α

Since the Transwell® experiments implicated soluble factors in mediating the inhibitory effect of primate stroma on porcine stem and progenitor cell growth, inhibitory cytokines were considered as potential mediators. TNF-α and TGF-β have been shown to have inhibitory effects on human hematopoietic cells (Van Ranst et al. 1996. *Experimental Hematology.* 24:1509; Bonnet et al., (1995) *Experimental Hematology* **23**:551). Previous studies in murine stromal cultures (Rogers et al. (1994) *The Journal of Immunology* **153**:4694; Waegell et al. (1994) *Experimental Hematology* **22**:1051 also showed an improvement in the growth of BMCs when antibody to either TNF-α or TGF-β was added, but there are no reports of their combined use. Figure 5 shows the results of the addition of either or both antibodies to cultures of porcine BMCs on a primate stroma in the presence of porcine pSCF + pIL-3. The addition of antibody to TGF-β alone showed a minimal increase in total CFU but not in cell numbers. With the addition of TNF-α alone there was a slight increase in total cell and CFU numbers. The addition of antibodies to both TGF-β and TNF-α produced the most striking effect as a marked enhancement of cell and CFU output was seen over the first three weeks of culture. The

combination of the two antibodies, therefore, seemed to have a synergistic effect.

As we have shown that a combination of blocking antibodies to both TNF-α and TGF-β dramatically increases the growth of porcine BMCs on a primate stroma over either antibody alone, same species cultures (primate BMCs on primate stroma and porcine BMCs on porcine stroma) with blocking antibodies were also assessed. Figure 6 depicts the results of adding these antibodies to the primate cultures in the presence of hSCF and hIL-3. The addition of either antibody alone to primate cultures produced a slight increase in total cell numbers and CFUs. A combination of both anti-human blocking antibodies showed no consistent improvement over either alone. When blocking antibodies were added to cultures of porcine BMCs on porcine stroma in the presence of pSCF and pIL-3, a relatively small effect on cell and CFU output was observed.

Overall, the addition of neutralizing antibodies to allogeneic cultures (primate BMC on primate stroma in Figure 6 and porcine BMC on porcine stroma in Figure 7) was unimpressive, especially when compared with the effect on xenogeneic cultures (pig BMC on primate stroma in Figure 5).

Since the blocking of TGF-β and TNF-α improved the growth of porcine BMCs on primate stroma, these antibodies were also evaluated in the mixed co-culture system. Results mirror the effects seen in the single cultures. A combination of the two antibodies shows an impressive enhancement in the survival of porcine BMCs when compared with those cultures where only one was added. The percentage of porcine BMCs and the numbers of CFUs recovered from

these cultures are shown in Table 1. At weeks 1 and 2 the percent of porcine BMCs and total CFUs from these cultures with both antibodies to TGF-β and TNF-α are higher than controls or single antibody cultures. At week 3, the percentage of porcine cells was increased in the co-cultures, especially in those with both antibodies added. Flow cytometric analysis also confirmed that the greatest percentage of porcine cells was in the cultures to which both antibodies were added.

Table 1. Growth of porcine BMCs on primate stroma in the mixed co-culture system

(a) % porcine cells by flow cytometry

Time after start of mixed co-culture (weeks)	Control (no antibody)	Addition of anti-TGF-β	Addition of anti-TNF-α	Addition of anti-TGF-β and anti-TNF-α
1	12	20	25	23
2	6.7	7.6	5.6	6.8
3	2.4	23.4	25.6	36.3
4	ND	ND	53.4	77

(b) Porcine CFU/well (%porcine CFU)

Time after start of mixed co-culture (weeks)	Control (no antibody)	Addition of anti-TGF-β	Addition of anti-TNF-α	Addition of anti-TGF-β and anti-TNF-α
1	187 (11.3)	250 (13.9)	409 (22.1)	595 (28.2)
2	99 (9.8)	115 (16.4)	194 (18.7)	499 (25.8)
3	7 (7.1)	27 (16.6)	236 (26.9)	307 (36.8)
4	ND	ND	63 (83.7)	256 (81)

Direct effects of human and porcine TGF-β and TNF-α on porcine and primate BMCs

To determine if differences in sensitivity to TGF-β and TNF-α exist between porcine and primate bone marrow cells both factors were added back to allogeneic cultures. Data are expressed as percent of controls. Two doses, 1 and 10ng/ml of human TGF-β were added to primate as well as porcine allogeneic cultures as shown in Figure 8. These results demonstrate that porcine BMCs are inhibited by huTGF-β to a much greater extent than primate cells are. Indeed, the low dose appeared to be stimulatory for primate cells. Figure 9 shows the effects of porcine TGF-β on primate and porcine cells. Results again show that porcine BMCs are more sensitive than primate BMCs to porcine TGF-β.

Shown in Figure 10 are the effects of human TNF-α on primate and porcine BMCs. Porcine cells were inhibited to a much greater extent than primate cells, similar to the results with TGF-β. Figure 11 shows the effects of porcine TNF-α on primate and porcine BMCs.

Results again show that pig cells were more sensitive to the inhibitory effects of porcine TNF-α. The results obtained from primate cultures show that porcine TNF-α appeared to be stimulatory, resulting in 2-fold increases in CFU production at 3 to 6 weeks.

5

EXAMPLE 2

Porcine Hematopoiesis in the Primate Microenvironment - *in vivo* Studies

Materials and Methods

Animals

Major histocompatibility complex-inbred (MHC-inbred) miniature swine are used in these studies. Baboons (Papio anubis, Biological Resources, TX) are recipients of the porcine BM.

Surgical procedures

All surgical procedures on pigs are performed under general anesthesia and have been described in Kirkman et al. (1979) *Transplantation* **28**:24. Before any surgical procedure, pigs receive a single dose of cefazolin (SmithKline Beecham Pharmaceuticals, PA) 10-20 mg/.kg i.m. A Hickman single lumen catheter is inserted into the external jugular vein to collect blood samples. Rib biopsies (fragments of ribs 3-4 cm long) are surgically removed as required for assays of bone marrow (BM). BM harvesting and processing techniques from pigs, for transplantation into baboons, have been described previously (Pennington et al. 1988. *Transplantation.*45:21). Anesthesia for surgical procedures on baboons (intravenous line insertion, splenectomy, BM biopsy) and supportive treatment have also been described previously Sablinski et al. (1995) *Xenotransplantation.* **2**:264.

All experiments are carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the

Subcommittee on Research Animal Care of the Massachusetts General Hospital.

Collection and processing of baboon BM

Baboon BM was collected over the course of several weeks before the experiment, whenever a myeloablative regimen is to be used. BM cells are depleted in vitro of CD2+ cells alone or CD2+ and CD20+ cells. Processed BM cells are frozen until infused into the baboon after completion of the conditioning regimen. More details are presented in Kozlowski et al., (1998) *Transplantation.* **66**:176.

Extracorporeal immunoadsorption (EIA) in baboons

Apheresis and perfusion of the plasma through immunoaffinity columns of synthetic Gal-α(1→3)-Gal containing trisaccharides (Alberta Research Council, Alberta, Canada) are used for removal of anti-pig natural antibodies. Details concerning these columns and their effectiveness in antibody removal have been described (Kozlowski et al. (1998) *Xenotransplantation.* **5**:122; Xu et al. (1998) *Transplantation.* **65**:172).

Conditioning regimens for baboons

The non-myeloablative conditioning regimen consists of (i) whole body irradiation (WBI) in two fractions of 1.5 Gy on day -6 and day -5; (ii) horse antihuman thymocyte globulin (ATG) 50 mg/kg i.v. daily on days -3, -2, -1; (iii) thymic irradiation (TI) of 7 Gy on day -1; (iv) splenectomy on day 0; EIA followed by porcine DM infusion on day 0 with subsequent EIAs and porcine BM infusion if necessary. WBI may be replaced by either higher doses of BM. TI may be replaced by antibody treatment. Costimulatory blockade molecules, e.g., CTLA4 Ig

and anti-CD154 (anti-CD40 ligand) antibody may be administered. Such procedures are described in Sykes PCT application WO 97/21836, Sachs US patent 5,876,708; Sykes PCT application WO 99/39726; Sykes and Sayegh, PCT application WO 99/39727), the contents of which references are incorporated entirely herein by reference. Pharmacologic immunosuppressive treatment may consist of cyclosporine (15-20 mg/kg/day for days 0-27 or longer as necessary).

Porcine cytokines as described in Kozlowski et al. (1999) *Xenotransplantation.* **6**:17-27 are administered as follows: porcine IL-3 and pSCF are administered at 100 μg/kg/day from days 0-14 and porcine GM-CSF is administered at 100 μg/kg/day from days 14-28.

Anti-TNF-α and anti-TGF-β antibodies are administered from days 0-14 at doses of approximately 10 mg/kg/day.

PCR for detection of pig DNA.

Detection of the porcine mitochondrial cytochrome B gene by polymerase chain reaction (PCR) is used for quantitative analysis of peripheral blood leukocytes or BMCs and for identification of porcine colonies in colony forming unit (CFU) assays on baboon BM samples. DNA is prepared from approximately 10^6 cells using a DNA isolation kit (QUIamp Blood Kit, Qiagen Inc. CA) and quantitated by Hoechst 33258 fluorescence on a Hoefer TKO 100 mini-fluorometer (Hoefer Instruments, CA). The protocol is more fully described in Kozlowski et al. (1999) *Xenotransplantation.* **6**: 17-27.

EXAMPLE 3

Comparison of the Use of LAP And Soluble TGFRII To Anti-TGF-β Antibody

In order to compare the blocking effects obtained using the anti-TGF-β 25 μg, latency-associated peptide (LAP, 100 ng, Cat. #. 246-LP-025, R&D Systems, Minneapolis, MN) and soluble TGF receptor (sTGFrII, 100 ng Cat. # 241-R2-025, R&D Systems, Minneapolis, MN) were added to cultures of porcine BMCs on a primate stroma. Results in Figure 12 show that LAP and sTGFrII improve the growth of porcine BMCs to the same extent as or better than anti-TGF-β antibody. These data indicate that the invention could be practiced with the use of these agents instead of anti-TGF-β antibody.

When the addition of soluble TNFrI (25 ng/ml, Cat. #. 225-B1-025, R&D Systems, Minneapolis, MN) & TNFrII (250 ng/ml, Cat. #. 226-B2-025, R&D Systems, Minneapolis, MN) is compared to the use of anti-TNF-α antibody results in Figure 13 show that the soluble receptors are just as effective as the antibody.

When considering a combination of inhibitors to TGF-β and TNF-α, just as was done with antibodies, results show that some combinations do work as well as both antibodies in cultures of porcine cells on a primate stroma. Combinations compared to anti-TGF-β and TNF-α were as follows; anti-TNF-α antibody and LAP; and sTNFrI&II and sTGFrII. Results in Figure 14 show that the addition of LAP to anti-TNF-α has an equivalent effect as compared to a combination of anti-TGF-β and anti-TNF-α antibodies. When soluble TNF receptors I & II and soluble TGF receptor II are added to cultures the resulting effects

are not improved over either added alone. The cause of this is most likely due to inadequate dosing of soluble receptors.

Since mixed co-cultures (porcine and primate BMCs on a primate stroma) more closely represent the *in vivo* model, the following combinations of inhibitors, anti-TNF-α antibody and LAP; and sTNFrI&II ("s" for soluble) and sTGFrII were added to mixed co-cultures to see if the effects were equivalent to or better than both antibodies. Results are depicted in Table 2. Growth of porcine BMCs with these combinations added is as good as if not better than both antibodies. These data suggest that the use of agents other than antibodies to block TGF-β and TNF-α may be useful in causing the synergistic growth of porcine BMCs in a primate environment.

Table 2. Growth of porcine BMCs in the presence of primate BMCs on a primate stroma

(a) % porcine cells by flow cytometry

Time post culture initiation	Control	anti-TNFα +TGFβ	TNFsrI&II +TGFsrII	LAP + anti-TNFα
1	24.81	16.94	25.06	9.6
2	5.86	8.30	5.58	15.03
3	2.62	4.31	2.51	3.53
4	1.32	2.47	3.24	2.48
5	1.46	3.13	1.16	2.26

(b) Porcine CFU/Well (% porcine CFU)

Time post culture initiation	Control	Anti-TNFα +TGFβ	TNFsrI&II +TGFsrII	LAP + Anti-TNFα
1	188 (23.1)	340 (27.7)	230 (32.5)	497 (20.1)
2	81 (6.4)	83 (16.0)	190 (8.1)	241 (20.9)
3	63 (5.6)	195 (21.2)	68 (4.0)	300 (12.1)
4	13 (1.9)	69 (12.7)	355 (34.7)	136 (72.7)
5	1 (0.8)	7 (17)	29 (21.6)	76 (24.6)

WHAT IS CLAIMED IS:

1. A process for enhancing engraftment of xenogeneic hematopoietic cells in a primate receiving xenogeneic bone marrow-derived cells, comprising administering to said primate, separately or as a mixture, an effective amount of a transforming growth factor beta (TGF-β) antagonist and an effective amount of a tumor necrosis factor alpha (TNF-α) antagonist.

2. The process of claim 1 wherein the bone-marrow-derived cells are hematopoietic progenitor cells.

3. The process of claim 1 wherein the bone-marrow-derived cells are peripheral blood stem cells.

4. The process of claim 1 wherein said administering to said primate follows administration of a conditioning regimen.

5. The process of claim 4 wherein said conditioning regimen is a myeloablative regimen.

6. The process of claim 5 wherein said myeloablative regimen comprises removal from said primate of a sample of bone marrow cells followed by *in vitro* depletion of either $CD2^+$ cells or both $CD2^+$ cells and $CD20^+$ cells from said sample, and subsequent reintroduction of said bone marrow cells back into said primate.

7. The process of claim 4 wherein said conditioning regimen is a non-myeloablative conditioning regimen.

8. The process of claim 7 wherein the non-myeloablative conditioning process is selected from the group consisting of whole

body irradiation, administration of horse anti-human thymocyte globulin (ATG), thymic irradiation, splenectomy, anti-T cell antibodies, co-stimulatory molecules and any combination of the foregoing.

9. The process of claim 8 wherein the co-stimulatory molecules are selected from the group consisting of anti-CD40 ligand antibody and CTLA4-Ig.

10. The process of claim 4 wherein said xenogeneic bone marrow-derived cells are hematopoietic progenitor cells.

11. The process of claim 1 wherein said xenogeneic bone marrow-derived cells are porcine cells.

12. The process of claim 1 wherein said primate is a human.

13. The process of claim 1 wherein said TGF-β antagonist is selected from the group consisting of anti-TGF-β antibodies, soluble TGF-β receptors, latency-associated peptide (LAP) and lysofilline, including active fragments thereof.

14. The process of claim 1 wherein said TNF-α antagonist is selected from the group consisting of anti-TNF-α antibodies and soluble TNF-α receptors, including active fragments thereof.

15. The process of claim 1 wherein said TGF-β antagonist is an anti-TGF-β antibody, or an active fragment thereof, and said TNF-α antagonist is an anti-TNF-α antibody, or an active fragment thereof.

16. The process of claim 15 wherein both antibodies are monoclonal antibodies or active fragments thereof.

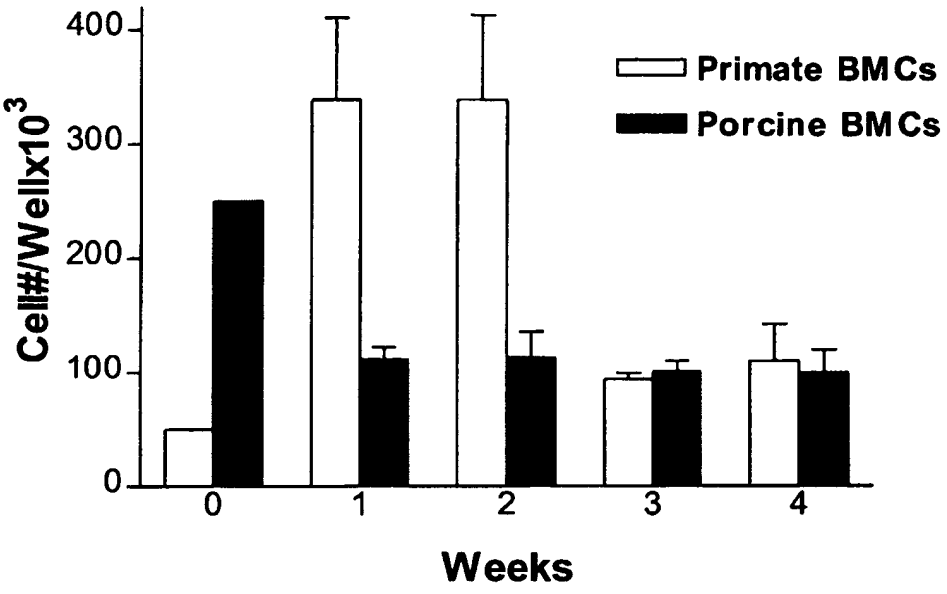
17. A composition comprising a therapeutically effective amount of a transforming growth factor beta (TGF-β) antagonist and a tumor necrosis factor alpha (TNF-α) antagonist.

18. The method of claim 1 further comprising the administration to said recipient of an effective amount of a cytokine.

19. The method of claim 18 wherein the cytokine is selected from the group consisting of interleulin-3 (IL-3), stem cell factor (SCF), and granulocyte-monocyte-colony stimulating factor (GM-CSF).

20. The method of claim 18 wherein the cytokine is porcine in origin.

Figure 1


400
300
200
100
0
Cell#/Wellx10^3
Primate BMCs
Porcine BMCs
0
1
2
3
4
Weeks

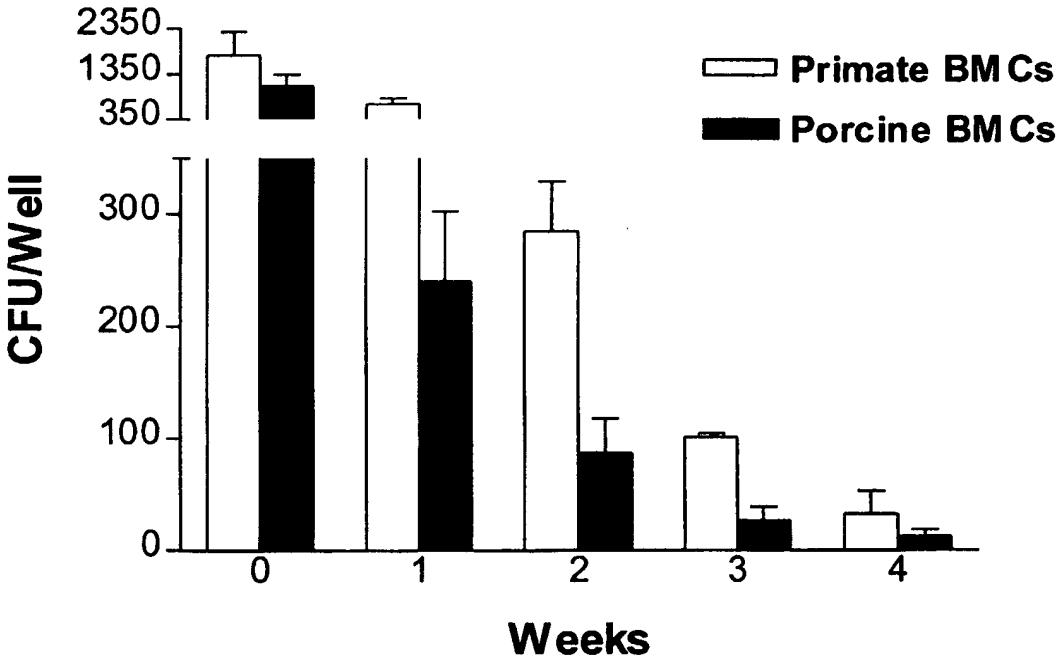
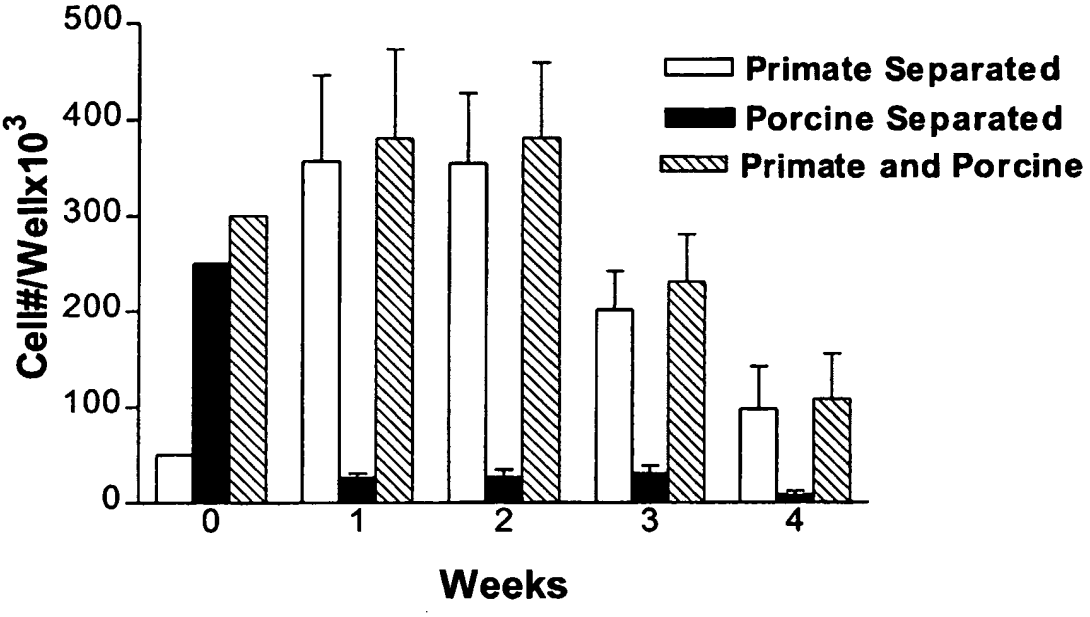

2350
1350
350
300
200
100
0
CFU/Well
Primate BMCs
Porcine BMCs
0
1
2
3
4
Weeks

Figure 2


500
400
300
200
100
0
Cell#/Wellx10^3
0
1
2
3
4
Weeks
Primate Separated
Porcine Separated
Primate and Porcine

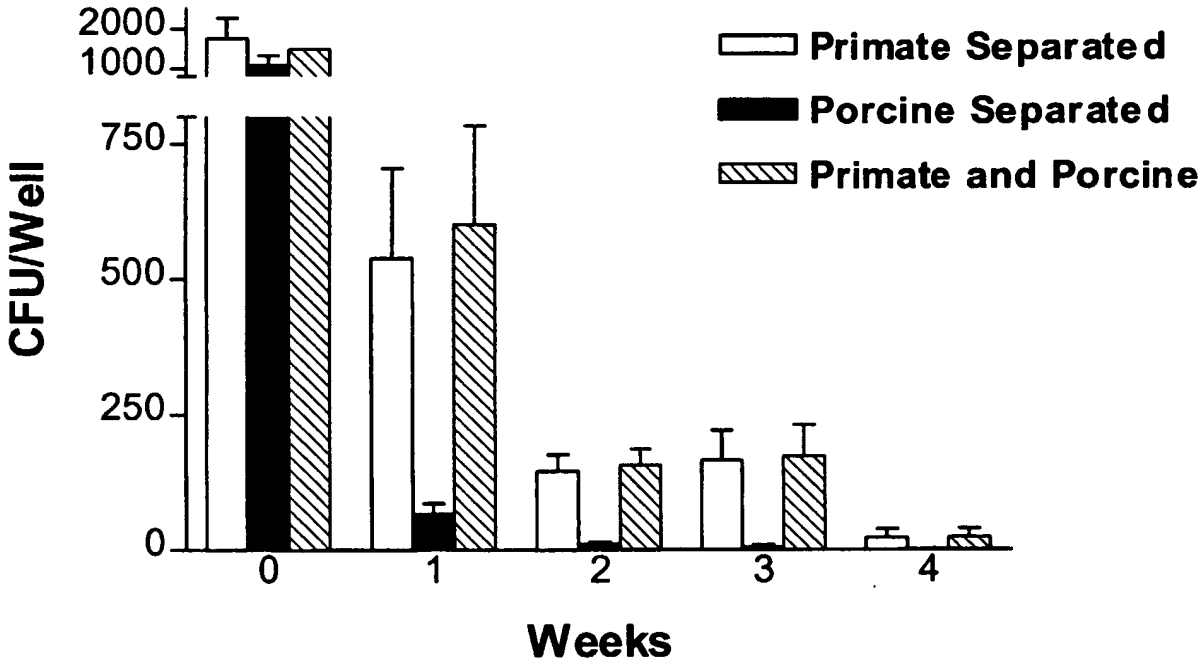
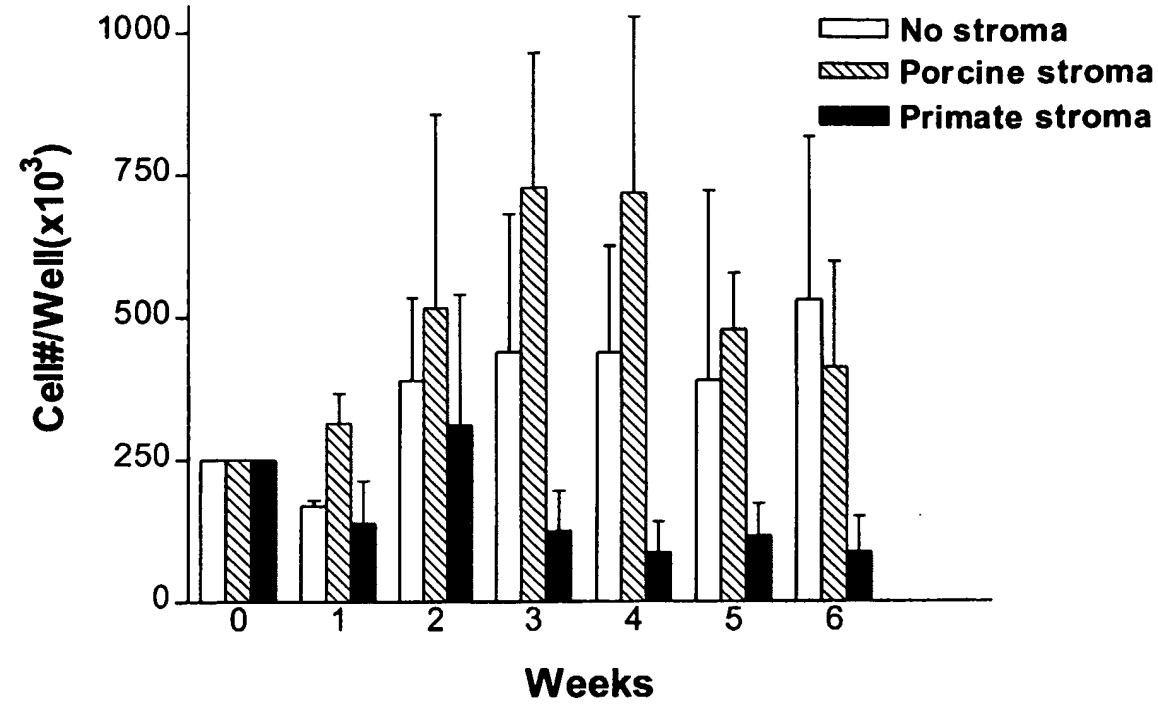
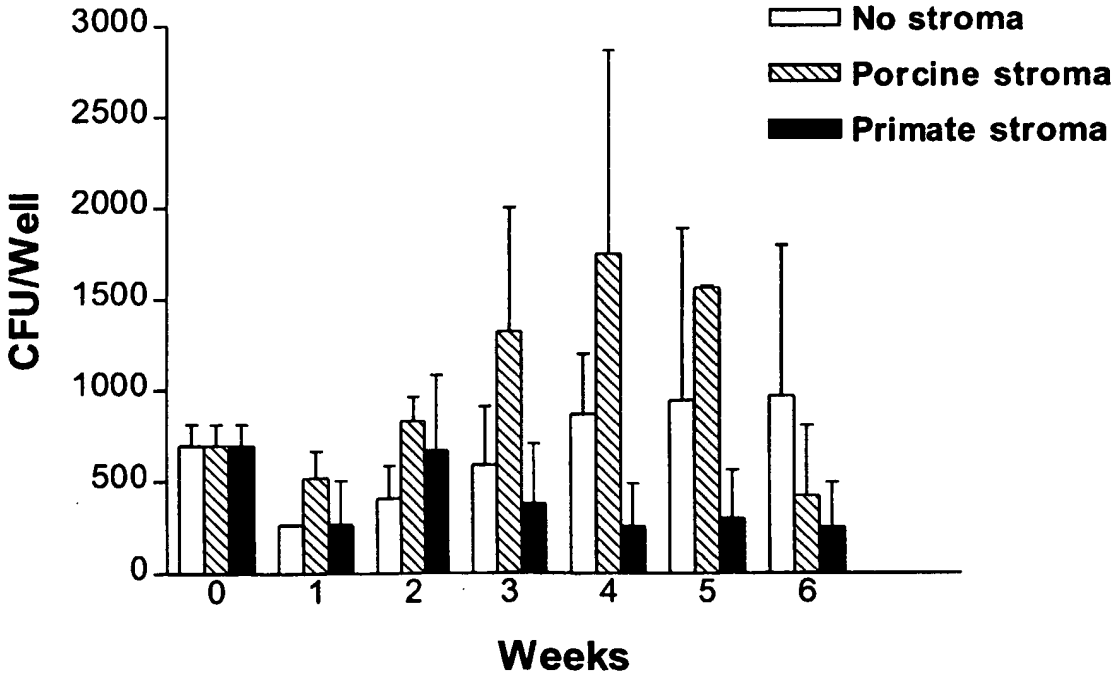

2000
1000
750
500
250
0
CFU/Well
0
1
2
3
4
Weeks
Primate Separated
Porcine Separated
Primate and Porcine

Figure 3


No stroma
Porcine stroma
Primate stroma
Cell#/Well(x10^3)
1000
750
500
250
0
0
1
2
3
4
5
6
Weeks


No stroma
Porcine stroma
Primate stroma
CFU/Well
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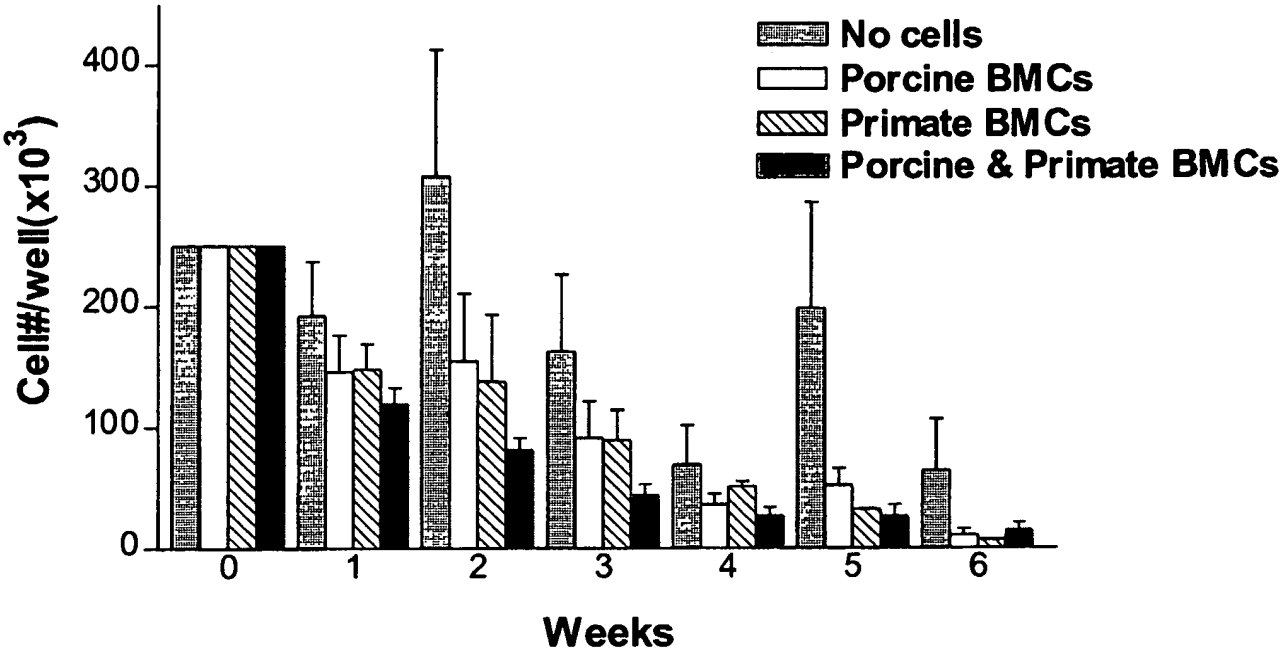
2500
2000
1500
1000
500
0
0
1
2
3
4
5
6
Weeks

Figure 4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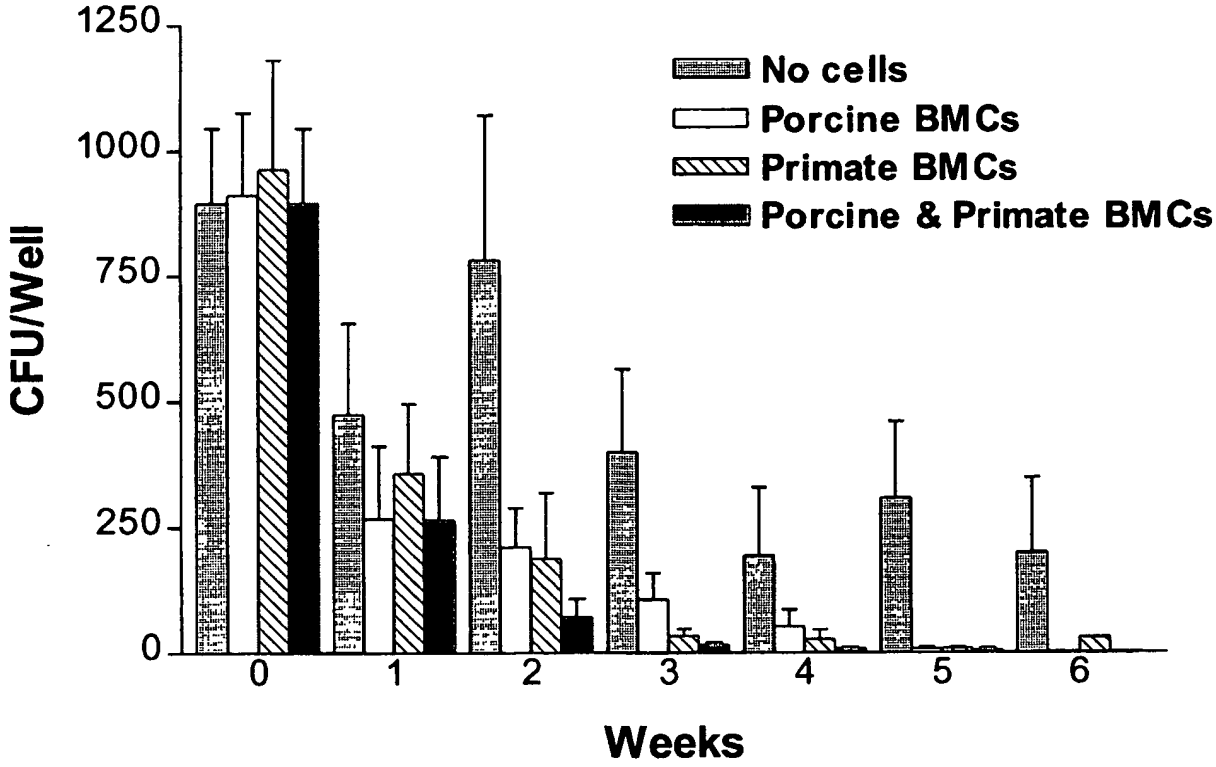
Porcine BMCs
Primate BMCs
Porcine & Primate BMCs
Cell#/well(x10^3)
400
300
200
100
0
0
1
2
3
4
5
6
Weeks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Porcine BMCs
Primate BMCs
Porcine & Primate BMCs
CFU/Well
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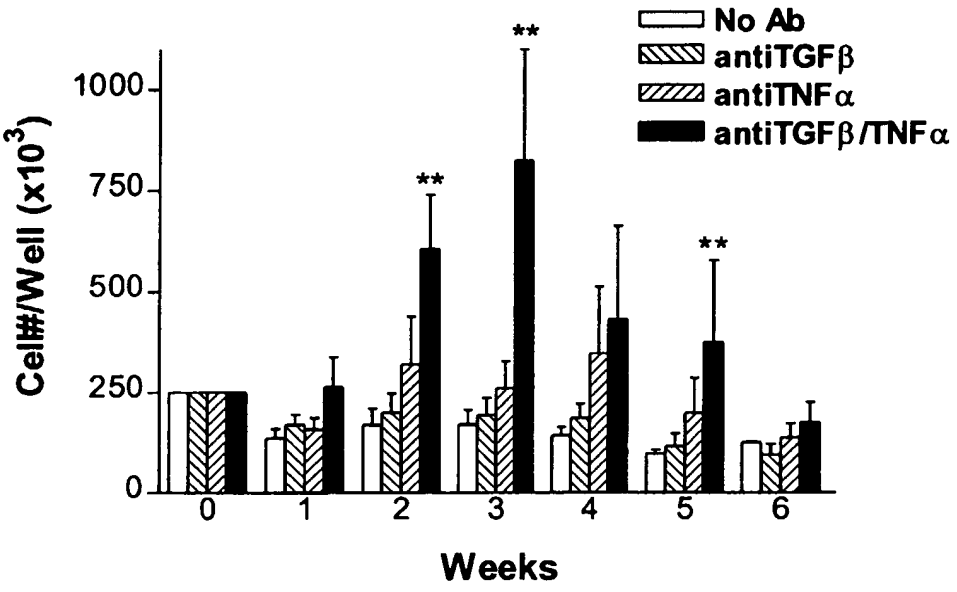
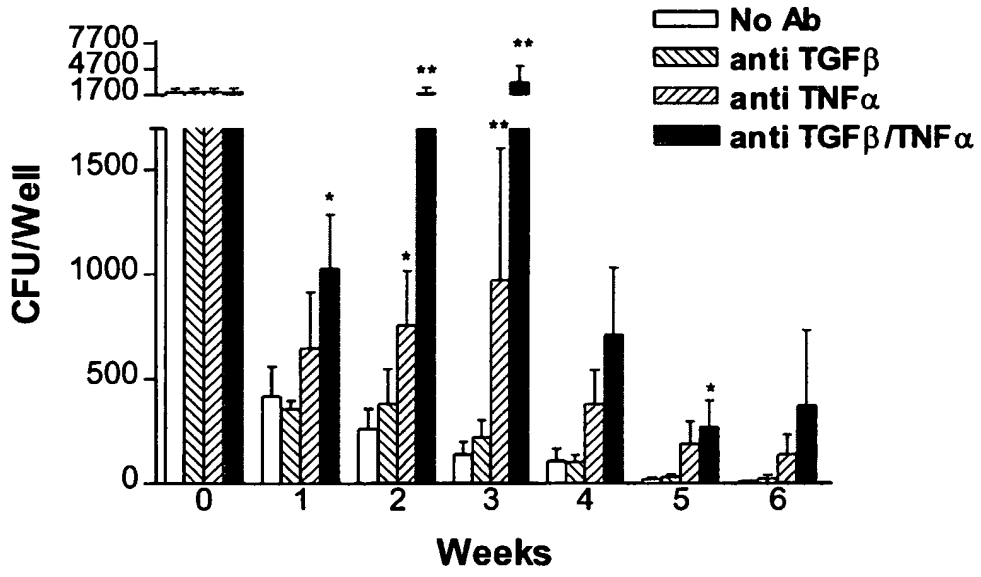
1000
750
500
250
0
0
1
2
3
4
5
6
Weeks

Figure 5


No Ab
antiTGFβ
antiTNFα
antiTGFβ/TNFα
Cell#/Well (x10^3)
1000
750
500
250
0
0
1
2
3
4
5
6
Weeks
**
**
**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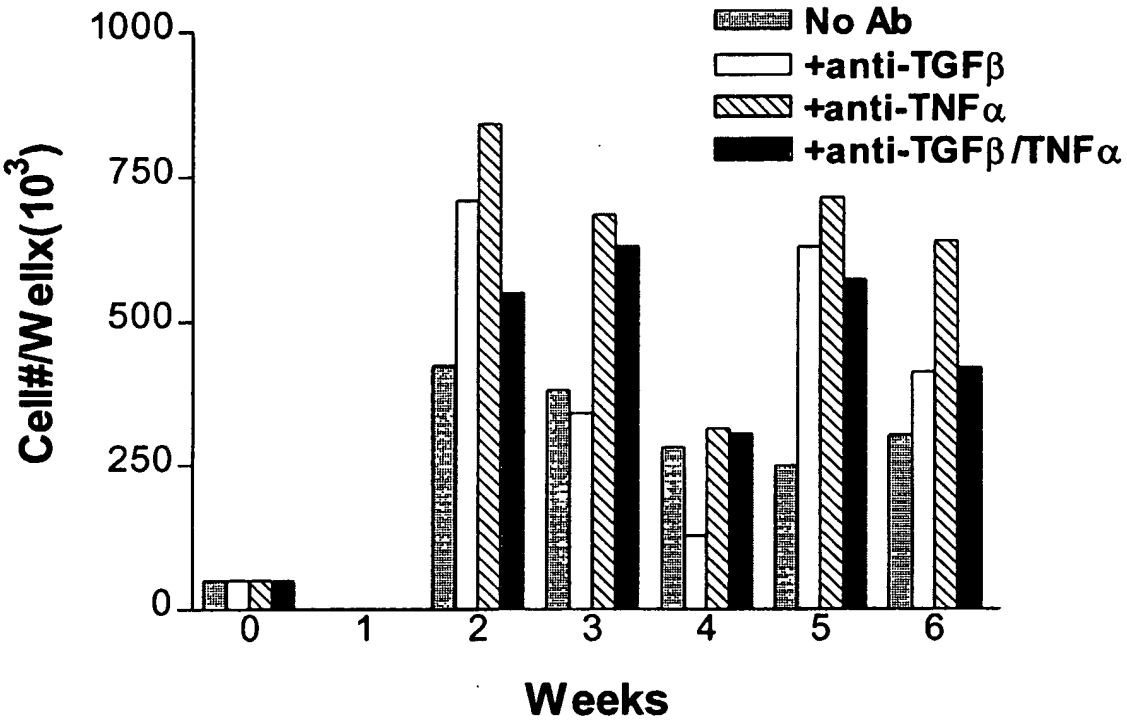
anti TGFβ
anti TNFα
anti TGFβ/TNFα
CFU/Well
7700
4700
1700
1500
1000
500
0
0
1
2
3
4
5
6
Weeks
*
*
*
**
**
**

Figure 6


No Ab
+anti-TGFβ
+anti-TNFα
+anti-TGFβ/TNFα
Cell#/Wellx(10^3)
1000
750
500
250
0
0
1
2
3
4
5
6
Weeks

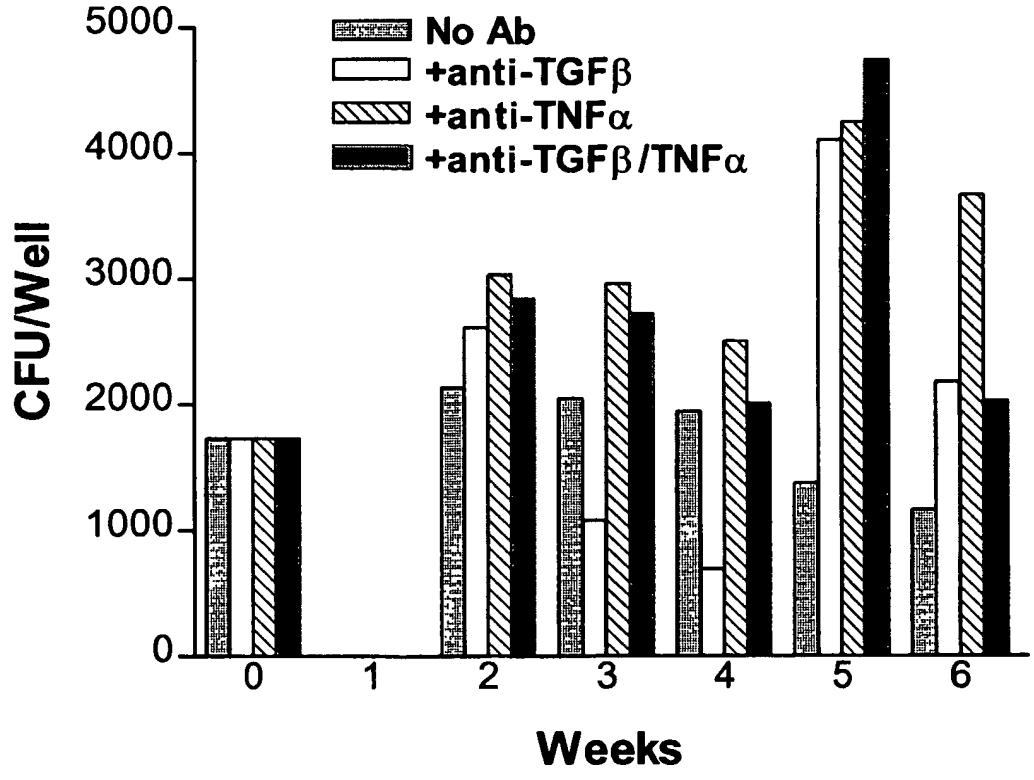
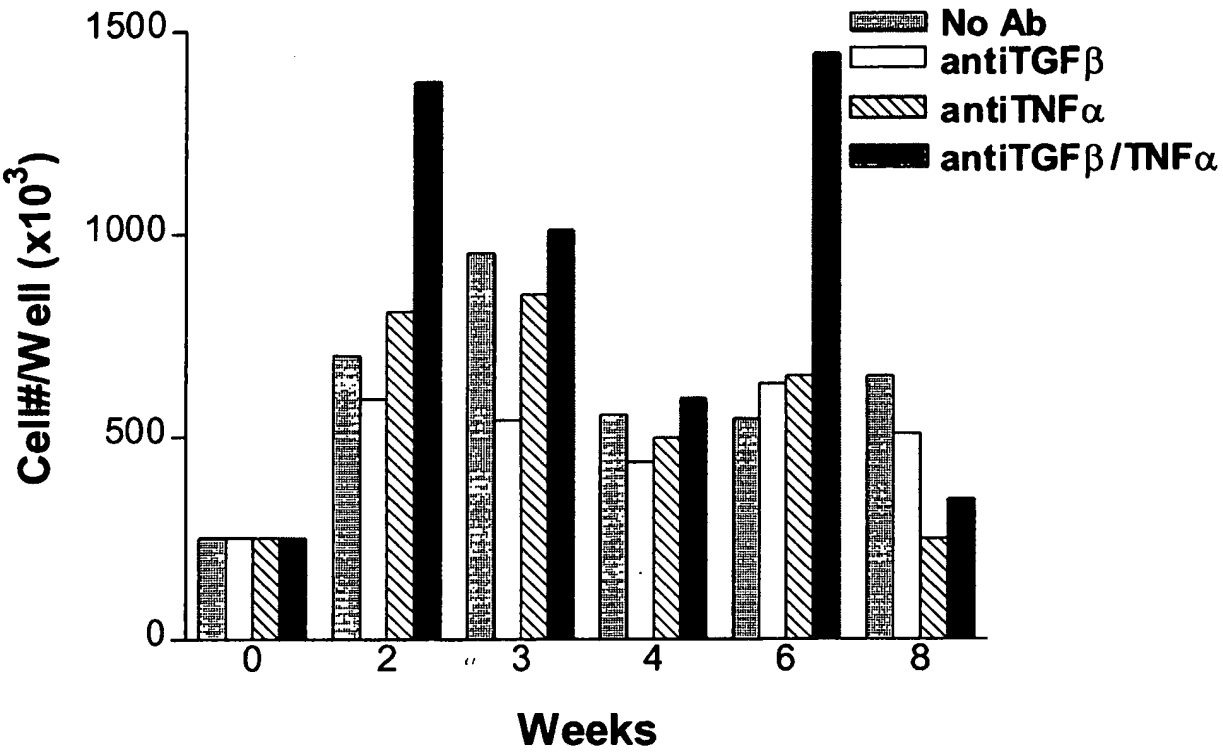

No Ab
+anti-TGFβ
+anti-TNFα
+anti-TGFβ/TNFα
CFU/Well
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
Weeks

Figure 7


1500
1000
500
0
Cell#/Well (x10^3)
0
2
3
4
6
8
Weeks
No Ab
antiTGFβ
antiTNFα
antiTGFβ/TNFα

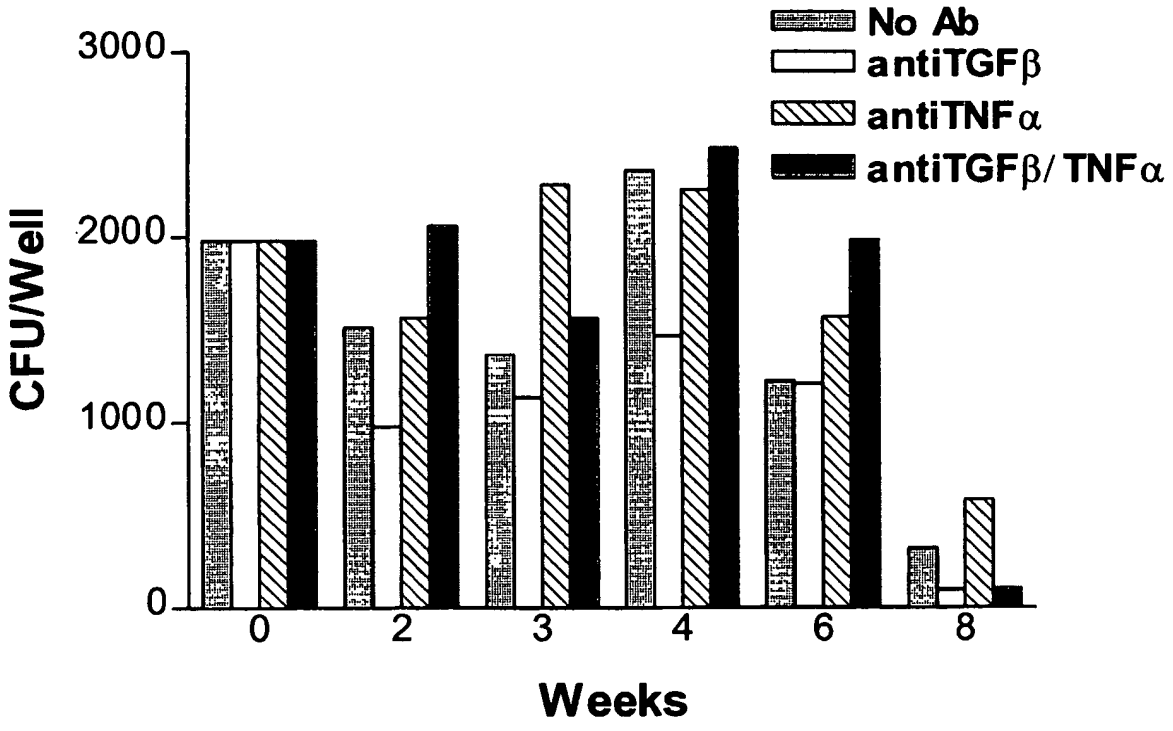

3000
2000
1000
0
CFU/Well
0
2
3
4
6
8
Weeks
No Ab
antiTGFβ
antiTNFα
antiTGFβ/ TNFα

Figure 8

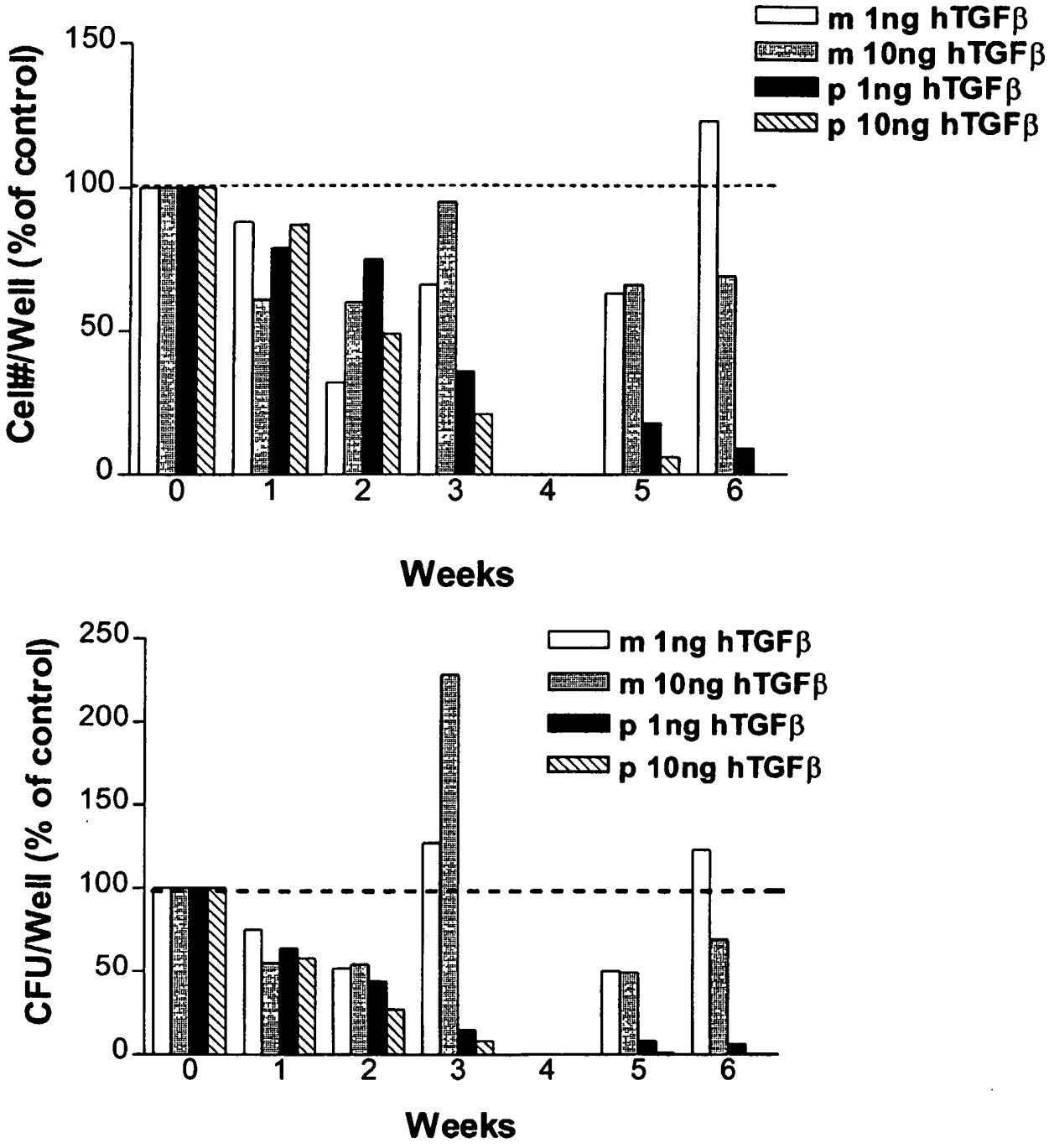
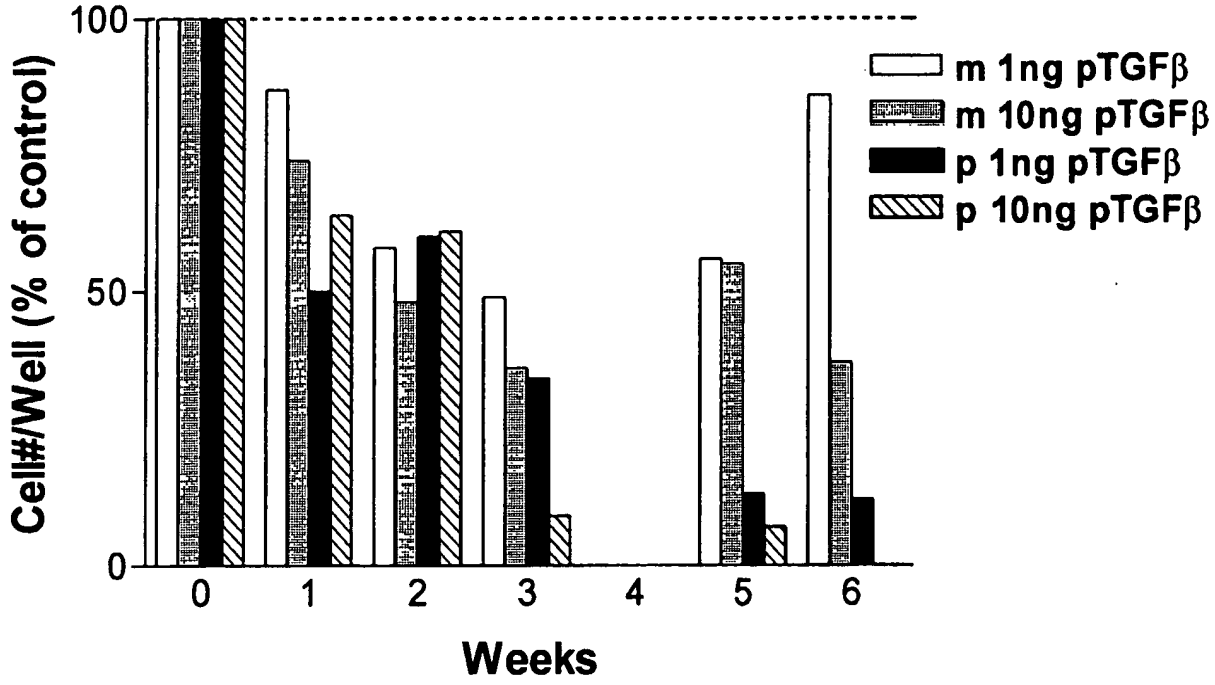

m 1ng hTGFβ
m 10ng hTGFβ
p 1ng hTGFβ
p 10ng hTGFβ
Cell#/Well (%of control)
150
100
50
0
0 1 2 3 4 5 6
Weeks
CFU/Well (% of control)
250
200
150
100
50
0
0 1 2 3 4 5 6
Weeks

Figure 9


100
50
0
Cell#/Well (% of control)
0 1 2 3 4 5 6
Weeks
m 1ng pTGFβ
m 10ng pTGFβ
p 1ng pTGFβ
p 10ng pTGFβ

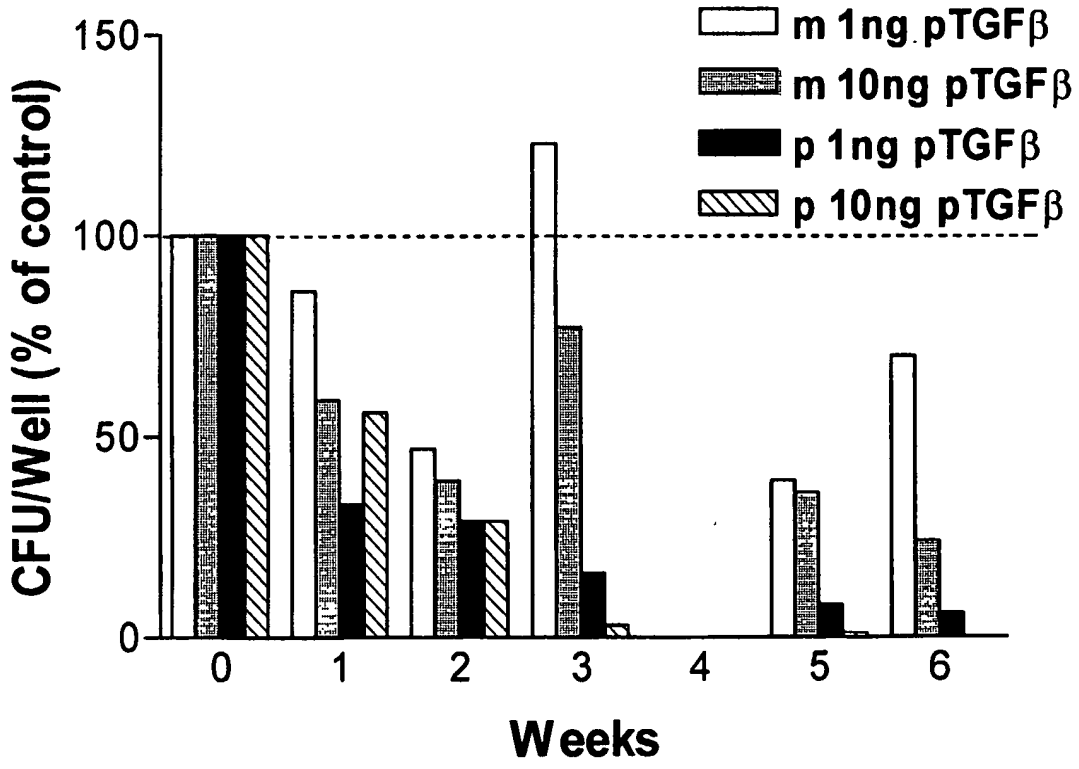
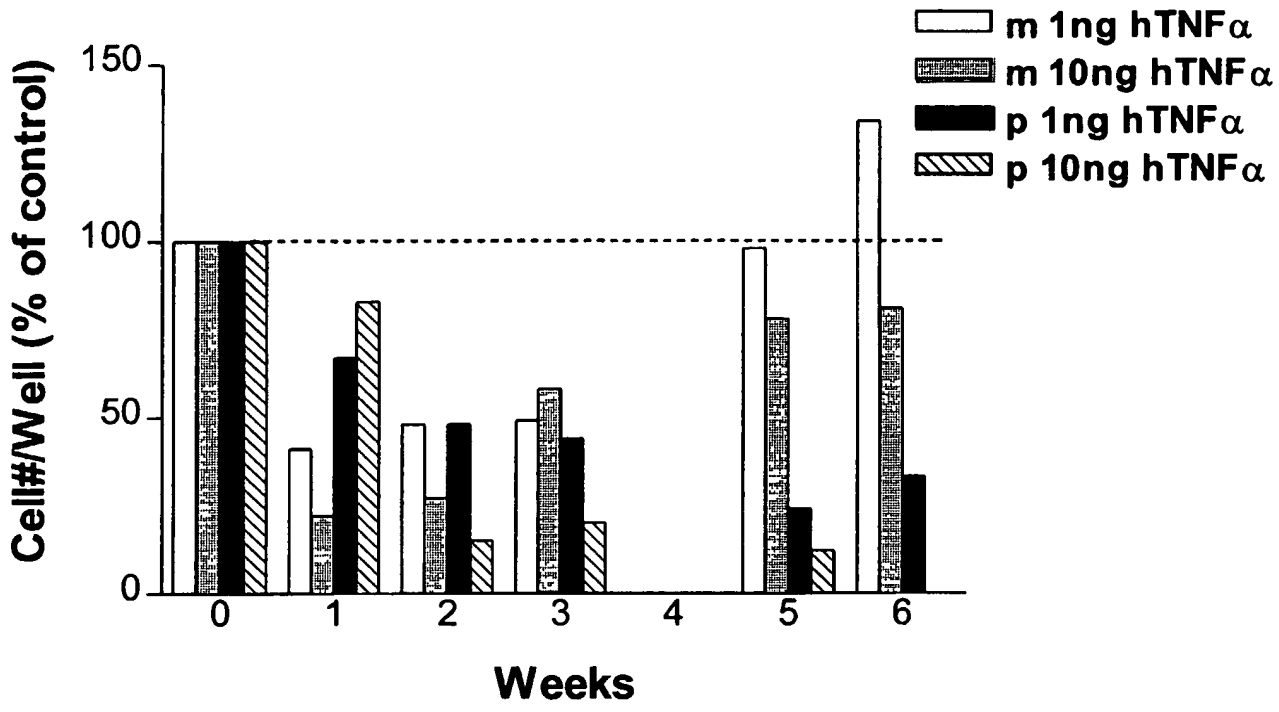

150
100
50
0
CFU/Well (% of control)
0 1 2 3 4 5 6
Weeks
m 1ng pTGFβ
m 10ng pTGFβ
p 1ng pTGFβ
p 10ng pTGFβ

Figure 10


m 1ng hTNFα
m 10ng hTNFα
p 1ng hTNFα
p 10ng hTNFα
Cell#/Well (% of control)
150
100
50
0
0 1 2 3 4 5 6
Weeks

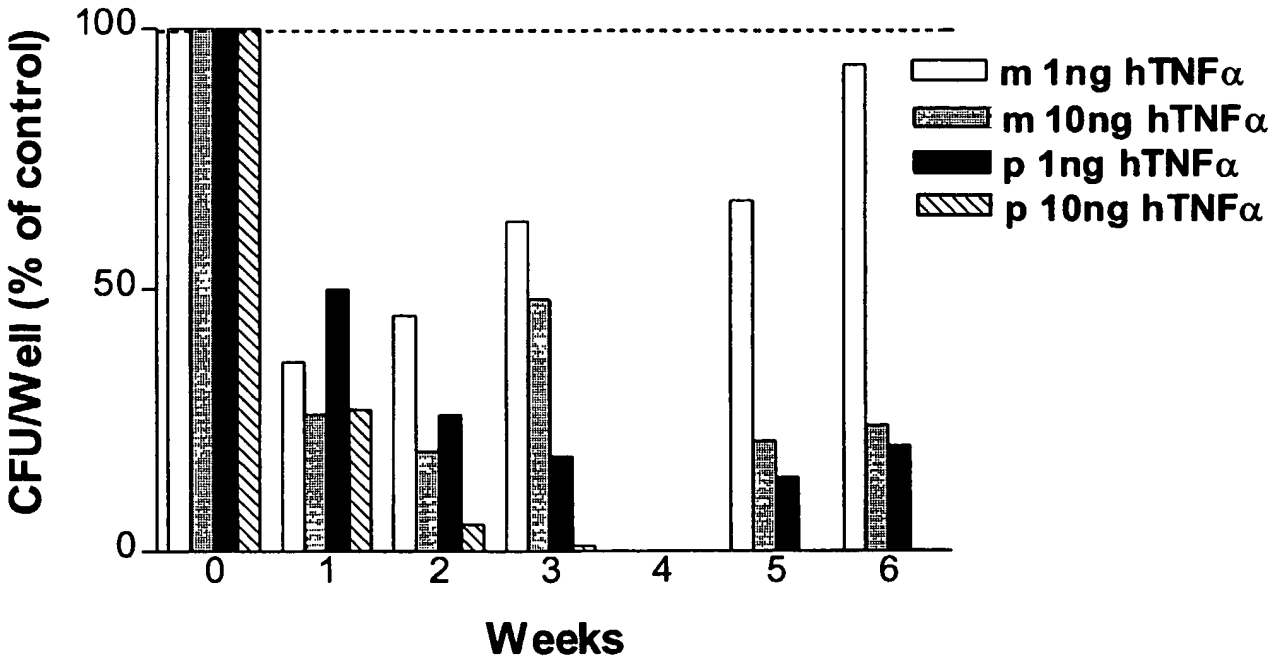
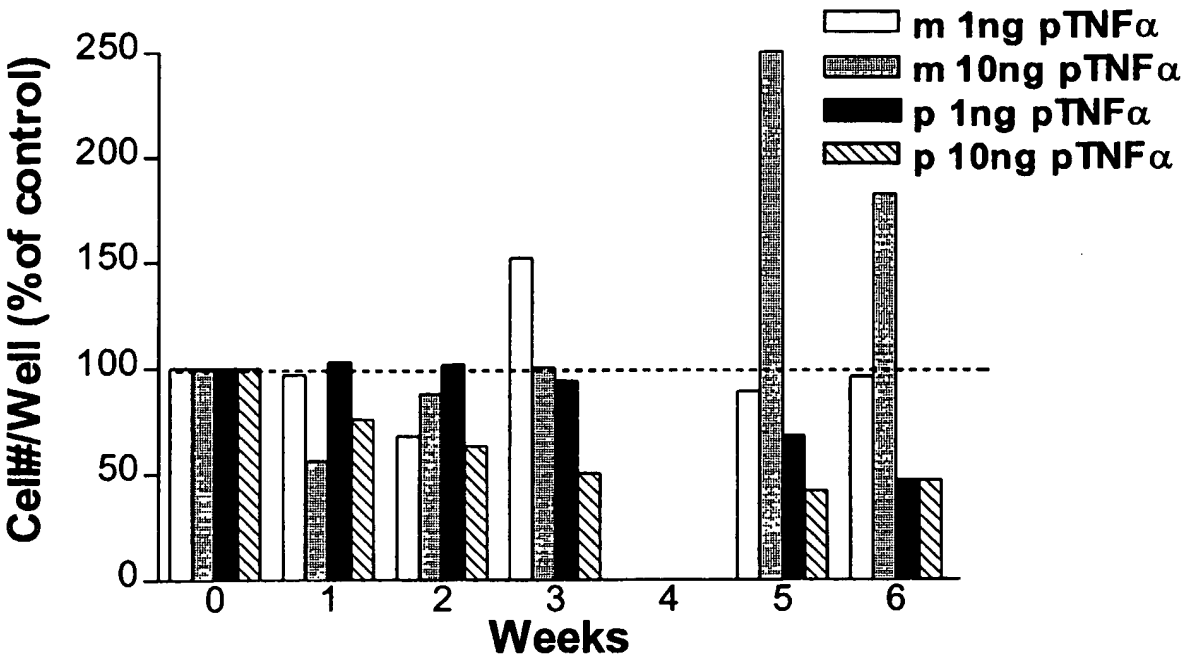

m 1ng hTNFα
m 10ng hTNFα
p 1ng hTNFα
p 10ng hTNFα
CFU/Well (% of control)
100
50
0
0 1 2 3 4 5 6
Weeks

Figure 11


m 1ng pTNFα
m 10ng pTNFα
p 1ng pTNFα
p 10ng pTNFα
Cell#/Well (%of control)
250
200
150
100
50
0
0 1 2 3 4 5 6
Weeks

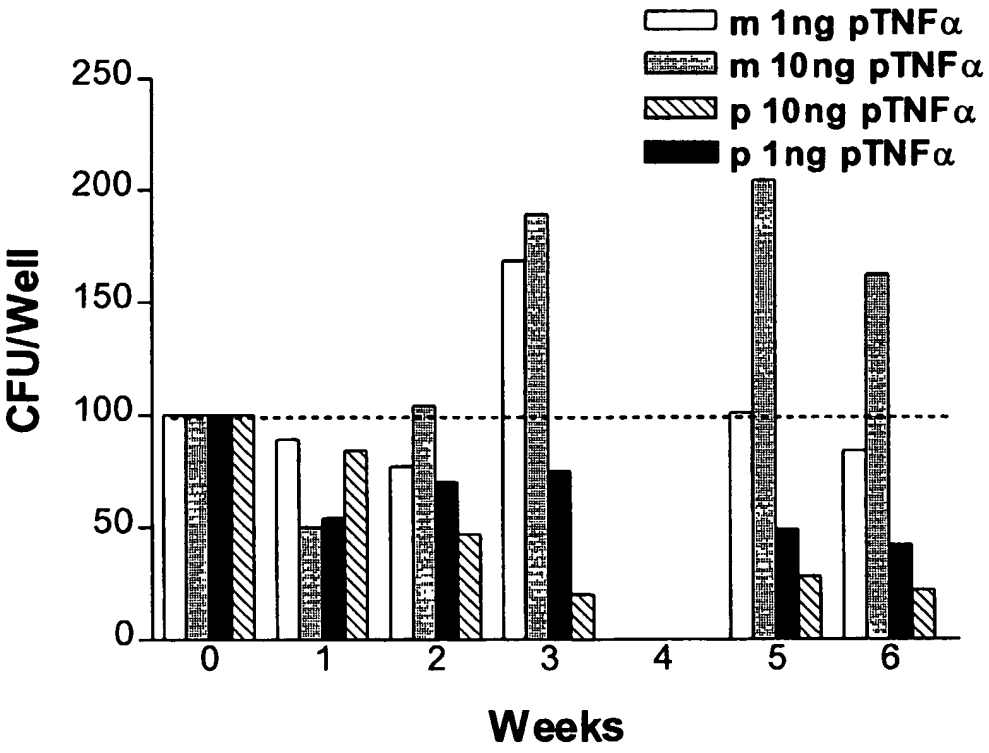
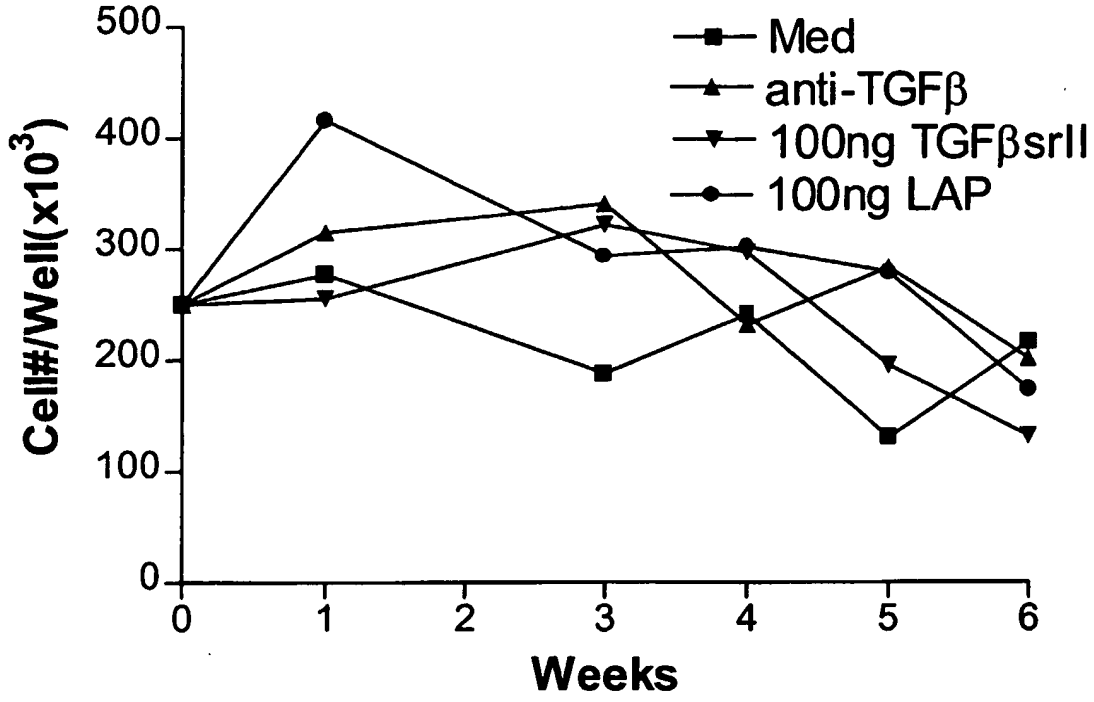

m 1ng pTNFα
m 10ng pTNFα
p 10ng pTNFα
p 1ng pTNFα
CFU/Well
250
200
150
100
50
0
0 1 2 3 4 5 6
Weeks

Figure 12


Med
anti-TGFβ
100ng TGFβsrII
100ng LAP
Cell#/Well(x10^3)
500
400
300
200
100
0
0
1
2
3
4
5
6
Weeks

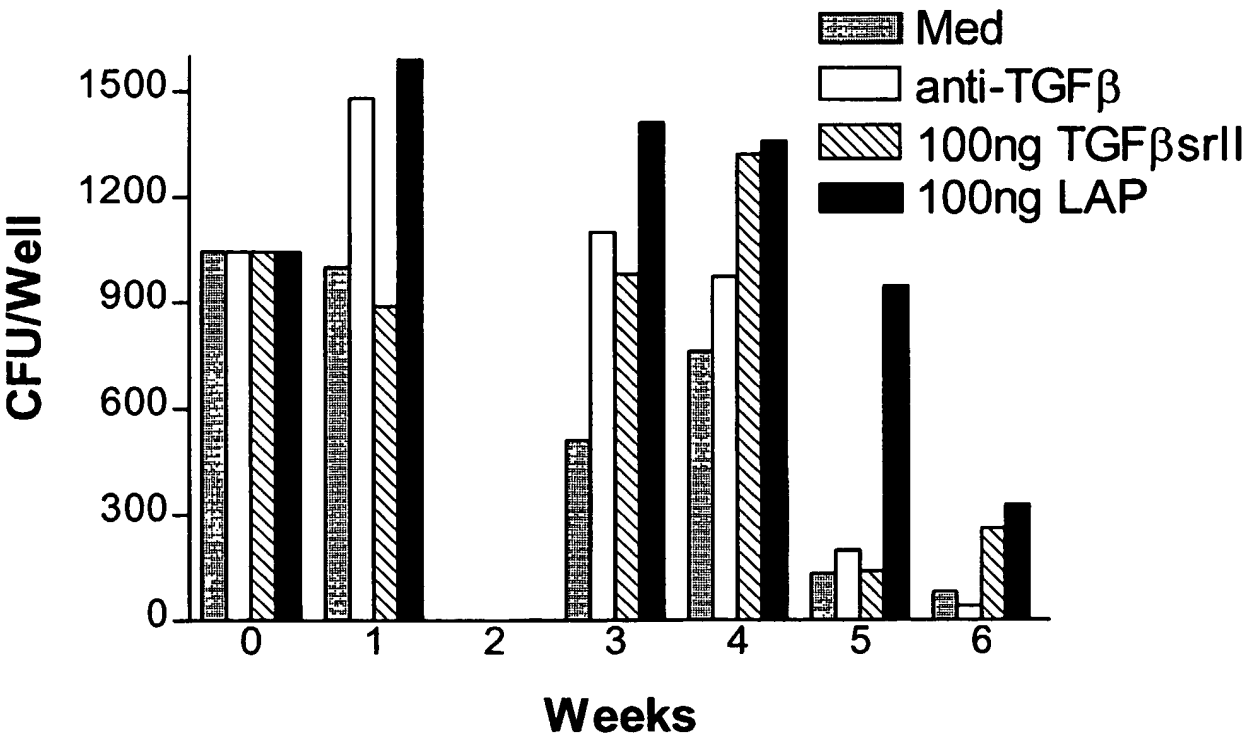
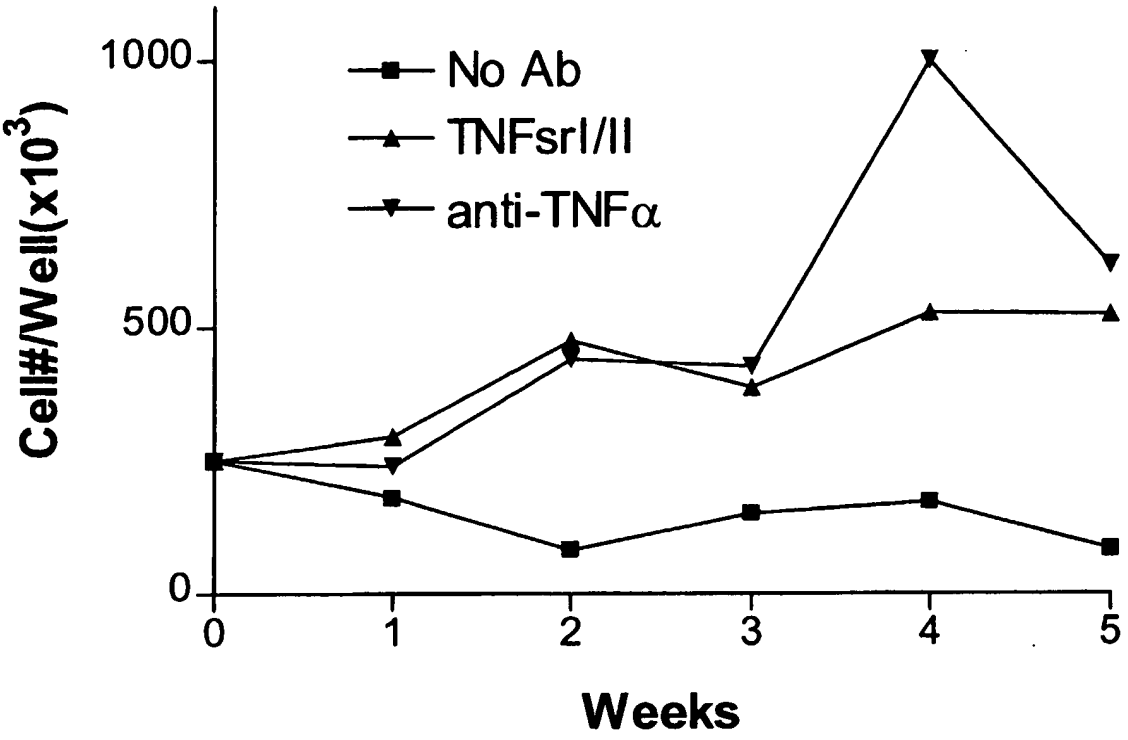

Med
anti-TGFβ
100ng TGFβsrII
100ng LAP
CFU/Well
1500
1200
900
600
300
0
0
1
2
3
4
5
6
Weeks

Figure 13


1000
500
0
Cell#/Well(x10^3)
No Ab
TNFsrI/II
anti-TNFα
0
1
2
3
4
5
Weeks

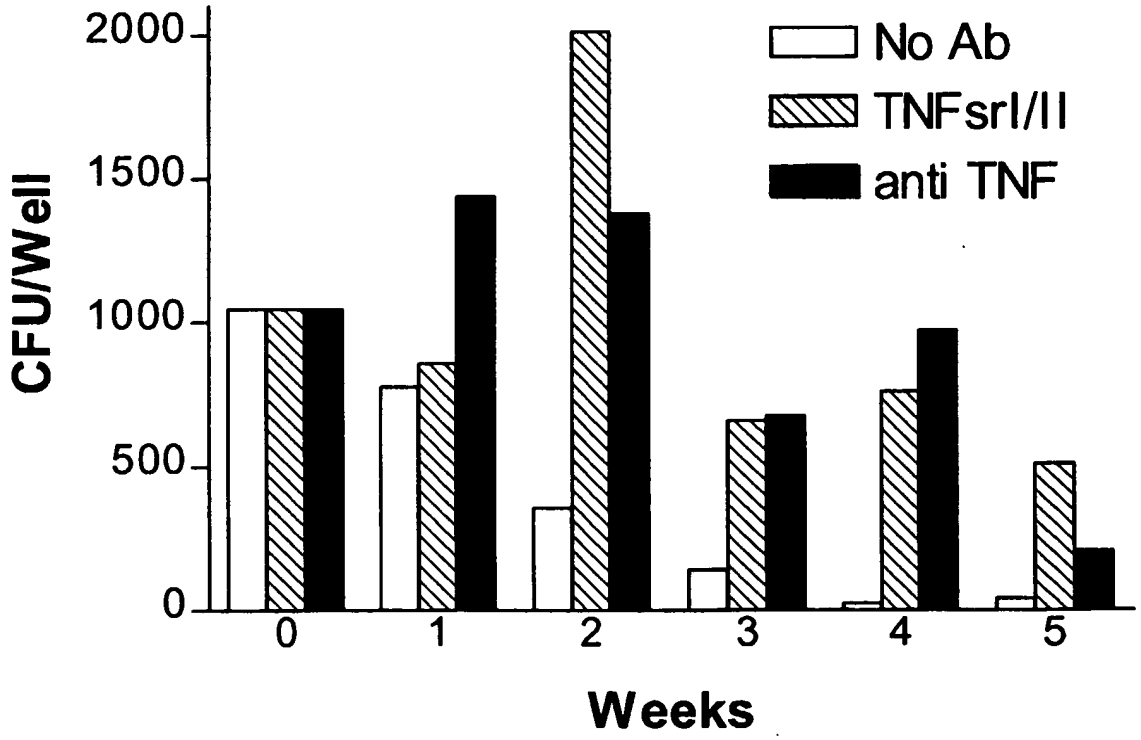
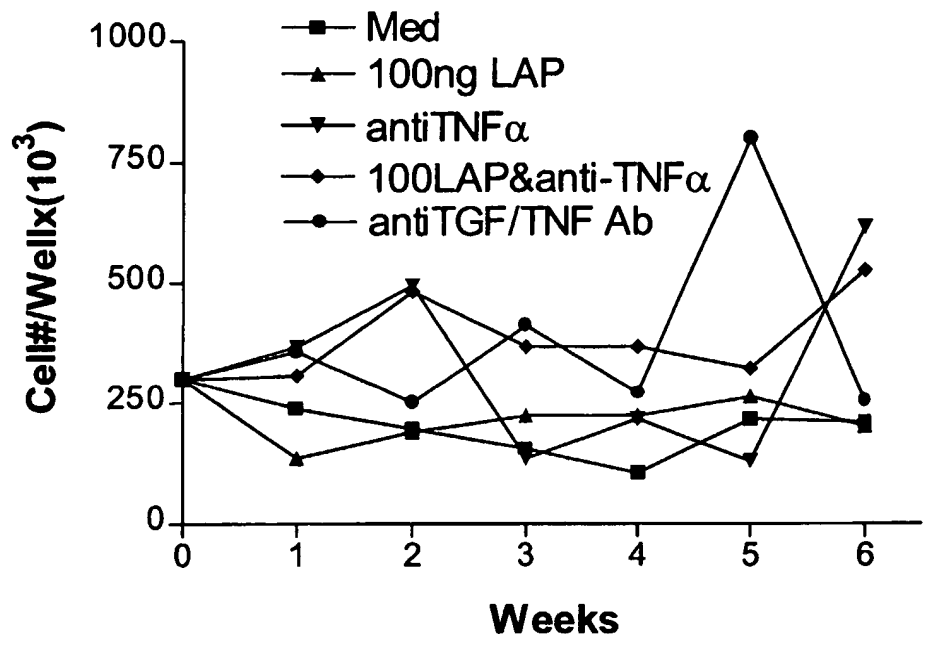
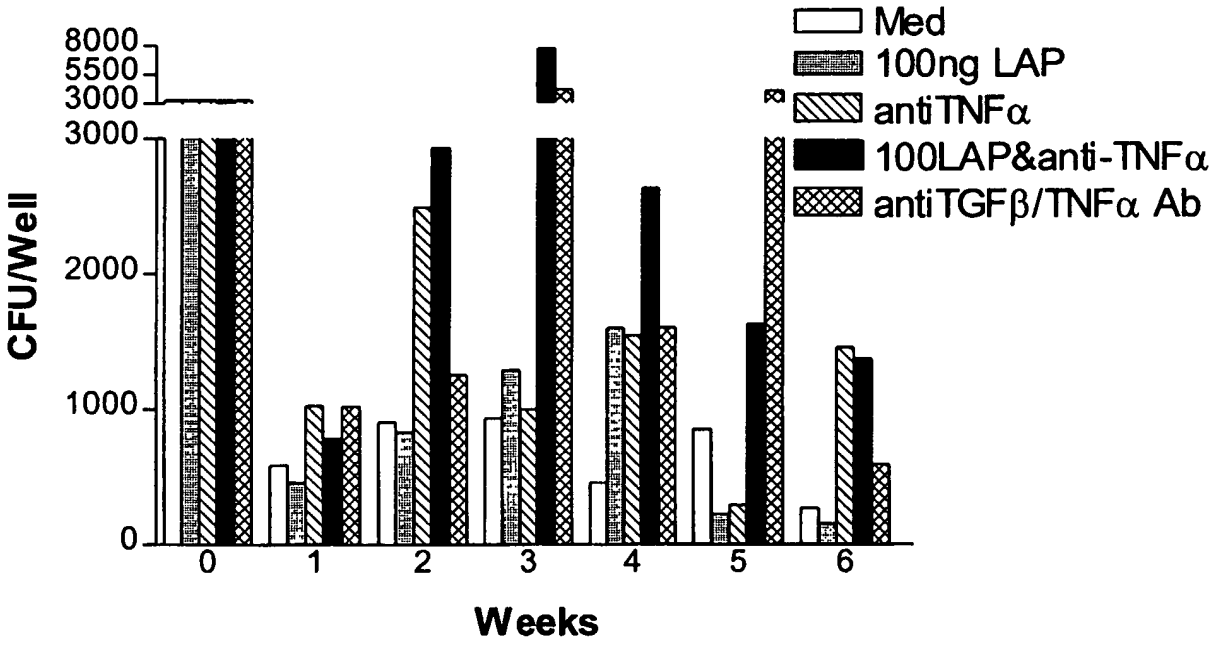

2000
1500
1000
500
0
CFU/Well
No Ab
TNFsrI/II
anti TNF
0
1
2
3
4
5
Weeks

Figure 14


Med
100ng LAP
antiTNFα
100LAP&anti-TNFα
antiTGF/TNF Ab
Cell#/Wellx(10^3)
1000
750
500
250
0
0 1 2 3 4 5 6
Weeks


Med
100ng LAP
antiTNFα
100LAP&anti-TNFα
antiTGFβ/TNFα Ab
CFU/Well
8000
5500
3000
3000
2000
1000
0
0 1 2 3 4 5 6
Weeks

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28982

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00, 39/395; A01N 1/02,
US CL : 514/2; 424/139.1; 435/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/2; 424/139.1; 435/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JACOBSEN, S.E.W. et al. Ability of flt3 Ligand to Stimulate the In Vitro Growth of Primitive Murine Hematopoietic Progenitors is Potently and Directly Inhibited by Transforming Growth Factor- Beta and Tumor Necrosis Factor-Alpha. Blood. 15 June 1996, Vol. 87, No. 12, pages 5016-5026, especially pages 5019 and 5021.	1-20
Y	EMERY, D.W. et al. Enhancement of Swine Progenitor Chimerism in Mixed Swine/Human Bone Marrow Cultures with Swine Cytokines. Experimental Hematology. August 1999, Vol. 27, No. 8, pages 1330-1337, especially pages 1332, 1333, and 1335.	18-20
Y	SABLINSKI, T. et al. Long-Term Discordant Xenogeneic (Porcine-to-Primate) Bone Marrow Engraftment in a Monkey Treated with Porcine-Specific Growth Factors. Transplantation. April 1999, Vol. 67, No. 7, pages 972-977, especially page 973.	18-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
14 December 2000 (14.12.2000)

Date of mailing of the international search report
31 JAN 2001

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/28982

Continuation of B. FIELDS SEARCHED Item 3: STN, EAST
search terms: xenograft, porcine, cytokine, TGF, TNF, antagonist